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IMMUNITY IN PLAGUE INFECTION

Results of 30 Years of Work with the "*Pasteurella pestis* EV" Strain (Girard et Robic)

by

G. Girard

Honorary Chief of Service,
Pasteur Institute
Former Director of the Pasteur Institute of
Madagascar (1922-1940)

Biologie Medicale (Medical Biology),
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INTRODUCTION

The third plague pandemia -- that of modern history -- which began in China in 1894 with the reawakening of the ancestral focus of the disease in Yunnan, and then spread by maritime routes in the five continents, is now on its decline. The 400 to 800 cases of Plague per year occurring in the world during the past five years are really insignificant compared with the hundreds of thousands of victims who have contracted this disease annually in British India alone between 1895 and 1930. This regression, which has assumed massive proportions especially during the last ten years, is due mainly to the application of a rational prophylaxis based on the fundamental discoveries of two French disciples of Pasteur: Yersin and Simond. In July 1894 Yersin isolated the causative agent in man and in the rat, and four years later Simond demonstrated its mode of transmission by the flea. However, certain natural conditions also played a role in bringing about this new situation which ought to make us adopt a more modest attitude; in ancient times these natural conditions were collectively referred to as the "epidemic spirit," and today we are still totally in the dark as to the nature of these conditions, as we also are regarding the processes which govern the genesis and evolution of the cyclic -- sometimes secular -- manifestations of the great epidemic plagues, interrupted for long periods of time by an apparent silence, while their

viruses* persist in certain animal species when they are exterior to man. This is the case of the zoonoses which include plague, whose perennial virus is maintained by rodents and their fleas. It is only by the whims of nature that we can explain the immunity which Europe enjoyed between the last two plagues which destroyed one quarter -- some say one half -- of its population in the 7th century (Justinian's plague) and then in the 14th century (black plague), at a time when a truly effective prophylaxis was unknown.

After Yersin's discovery and in view of the rapid spreading of plague, great hopes were attached, very prematurely, to preventive vaccination. The latter was employed everywhere, with more or less beneficial results, the vaccines being composed of suspensions of germs killed in various ways which we shall not discuss at this point. However, their practical value soon became a subject for debate, and while there was no reason to deny that these vaccines were effective to some extent, the populations who were obliged to undergo vaccinations nevertheless were aware of the lack of success. We have discussed this problem in 1936 [18], when we treated the difficulties and possibilities of the immunization of man against plague, the information obtained from animal experiments, and the interpretation of the vaccination results as a function of the epidemiological factors, and concluded that it was necessary to strengthen the experimental control of the vaccines designed for human protection. Our observations made at Madagascar, and those of L. Otten gathered at Java, led us to recommend, and substitute for the vaccines then in use, a live vaccine (a *virus-vaccine*, in the Pasteurian sense of the term) designated by the symbol EV**. For the past 30 years this strain of *Pasteurella pestis* has been the object of investigations in many French and foreign laboratories. Until about 1940, the major and immediate objective of these studies was the immunization of the populations living in an endemo-epidemic environment, particularly in Madagascar. During and after the World War, in the light of knowledge acquired on the antigenic composition of the pathogenic bacteria which only incidentally touched upon Yersin's bacillus, this microorganism has become the object of a series of investigations in the US, in Great Britain and in the Soviet Union, investigations which include the study of the EV strain. The latter is now well known and its study has contributed to the clarification of certain aspects of antiplague immunity. However, this study is scattered through many publications, and we have considered it

*This term is used in the broad sense, comprising all agents of the infectious diseases.

**The first two letters of the name of the child possessing the bubo from which the strain was isolated in 1926.

expedient to collect all that is known about this strain into a single synthetic work in which, as we go on, we shall express our opinion on debatable material in the most objective manner possible.

Consequently, this article will be divided into two parts: the first will relate to the discovery of the EV virus-vaccine, to the basic experiments which have demonstrated its innocuous nature and its protective effect in the animal, and its applications to human vaccination; the second part will treat the processes taking part in the immunity to plague in correlation with the antigenic structure of its etiological agents and, by extension, that of the EV strain.

To conclude, we shall report on a curious finding, unique in microbiology until now, even though it has no connection with immunology: the production by the EV strain, and by this strain alone, of crystallomorphic concretions whose meaning is completely unknown to us.

Part I.

THE EV VIRUS-VACCINE AND ITS APPLICATIONS

How We Were Led to Recommend the Use of a Live Vaccine Against Plague in Madagascar

Two observed facts -- one of an epidemiological and local nature, and the other, experimental -- have oriented us toward this new approach.

1. Having made contact, in Tananarive in February 1922, with plague which had manifested itself here for the first time six months earlier, and which had encountered a set of conditions which were propitious for its implantation and spreading in the whole Plateau region [39], we followed the course of this advance from year to year; the disease spread despite the laudable efforts made by the administrative and public-health authorities to extend vaccination to all the inhabitants of the infected districts. In truth, the only prophylaxis which had limited the extension of foci of infection was that which was applied against the virus reservoir -- rats and fleas -- but from which nothing could be expected outside certain relatively large centers. It was sufficient to penetrate into one of the innumerable hamlets scattered over the Malagasy countryside to realize that the inhabitants lived there under conditions which could not have been too far from those of our own peasants at the time of the pandemia of the Middle Ages. The problem of human plague -- let us not forget -- is essentially a problem of dwellings. Hence, only an efficacious vaccination offered the hope of reducing the morbidity which continued to assume disquieting proportions, with a high mortality rate due, for the most part, to episodes of pneumonic plague combined with the classical bubonic forms, episodes which, through direct interpersonal contagion, sometimes led to the disappearance of entire families*.

*It is only since the advent of certain funcal antibiotics (especially streptomycin) that, since 1947, primary pneumonic plague, which up to then had been irremediably fatal, has become easily curable, as have all the other forms of plague.

2. Experiments repeatedly carried out between 1922 and 1926 in an attempt to confer an appreciable immunity on sensitive rodents revealed that while the rat and the mouse benefited from a certain degree of protection when treated with killed vaccines, vaccination constantly failed in the case of the guinea pig, regardless of the type of vaccine employed. This contrast did not cease to intrigue us. Moreover, the control tests of these vaccines tended, above all, to prove their sterility and their innocuousness: to our knowledge no test had ever been required before to prove the effectiveness of these vaccines in the animal. If we refer to the study of Dujardin-Beaumetz [31] published in 1912 when millions of vaccinations had already been performed, we read that "the control inoculations carried out in the laboratory are always more severe than under the natural conditions of contagion. Moreover, the experimental animals such as monkeys, rats, mice and *especially guinea pigs* (our italics) exhibit an extreme sensitivity to the plague virus, and one cannot count on these data to determine the time when the vaccinated human is protected from a plague attack." The least that one can infer from the opinion expressed by our esteemed teacher who has often confirmed this to us later on, is that antiplague vaccination, which was begun with the speed of lightning especially in India with the use of Haffkine's lymph, had not been based on solid experimental foundations. However, its effectiveness was indicated by statistics relating to millions of vaccinated subjects, and this alone was sufficient for recommending its use and for insisting on the necessity of imposing it.

Fig. 1. Public-Health Team About to Remove the Body of a Victim of Pulmonary Plague at Madagascar (Photo: Pasteur Institute, Tananarive).

At Tananarive we maintained, by regular subculture, the strains of plague virus which we isolated from man or from the rat, all of which were, originally, highly virulent for the guinea pig. After certain periods of time ranging from a few months to 2-3 years, several of these strains were found to be highly attenuated in their pathogenic power: the guinea pig survived after reacting solely by an inflammatory lesion at the inoculation site, sometimes accompanied by a satellite adenopathy, which was over in about 20 days with or without suppuration, apparently without the general state of health of the animal having been affected. If during the following two months we subjected these guinea pigs to a severe virulent test, they supported it without damage. After killing these animals a few weeks later we found neither an adenopathy nor a macroscopic visceral lesion in them, and the inoculations did not reveal the presence of any *Pasteurella pestis* in the tissues. Hence, it was possible by means of a germ of weakened virulence to confer a high degree of immunity on the guinea pig; this was a fact duly established in 1926. Since then our objective has been to find a strain which would meet the requirements of an eventual immunization of man, namely, innocuousness and efficacy. Six years elapsed before this objective was attained.

Experimental Research and Data Obtained (1926-1932)

Parallel to the attenuation of the pathogenic power of the plague bacillus by serial subculture in nutritive agar, we attempted to speed up this attenuation by several procedures: culture in broth treated with beef bile, alcohol, extracts of organs of refractory animals such as the tanrec (*Centetes ecaudatus*), a Malagasy insectivorous animals, whose natural and total immunity to experimental plague we have proved earlier [38a], or filtrates of pyocyanic bacilli known for having been successfully employed by certain authors on other pathogenic microorganisms. In fact, these expedients were abandoned in view of the findings that by comparison the best and most consistent results were obtained by means of simple monthly subcultures at laboratory temperature (about 20°).

Having available, in this manner, several strains which exhibited a protective effect in the guinea pig, the task now was to find out whether this immunity was not merely a firm but also a durable immunity. The animal experiments with killed vaccines had not been sufficiently conclusive to enable us to obtain any reliable references from the pertinent literature. As for the vaccination of man, it was assumed that an immunization by means of two injections given one week apart did not prolong the effect of these injections beyond 5-6 months, and that revaccinations were recommended for persons exposed to a permanent risk of infection.

Our studies, spread over long months, convinced us that while all strains of *P. pestis* which have achieved a certain degree of attenuation were endowed with a high protective power when the guinea pigs were tested 2-3 weeks after the inoculation, nevertheless the various strains exhibited considerable variations with respect to the duration of the immunity conferred in this manner.

Note. Let us describe, once for all, the nature of the virulence test to which our guinea pigs were always subjected during the control of the value of a live vaccine: this is indispensable in order to avoid errors in the interpretation of what we have denoted in this article, for the sake of convenience, as total immunity (TI), partial immunity (PI) and absence of immunity (AI).

The skin of the flank of the guinea pig is depilated, shaved and slightly excoriated on an area of 5 to 6 cm^2. The spleen of a mouse which has died of septicemic plague is ground with fine sterile sand and suspended in 10 ml of physiological saline solution; the skin surface in question is rubbed for several seconds with a wad of hydrophilic cotton dipped into the suspension. This technique is based on the 60-year-old procedure of Albrecht and Ghon, which has been used for the post mortem tracking down of plague which we have put into practice in Madagascar in 1922 [40].

The severity of such a test is undeniable, because a mouse spleen affected by plague contains several billions of very virulent germs [41]. Hence, we cannot say how many lethal doses this test corresponds to, but it corresponds to at least several thousand such doses, because on decimal dilution of the parent suspension employed, the guinea pig or mouse is still killed as a result of acute plague when a 10^{-7} dilution is cutaneously administered [42]. Without claiming that our animals have always received 10 million lethal doses, we are in a position to state that the severity of the test was by far greater than that of a natural infection. Hence, when we state that in the vaccinated guinea pigs the immunity has weakened or disappeared after time "t " this is to be understood in terms of the test defined above. This does not necessarily imply that the animals are deprived of any protection compared with the inoculation of a few lethal doses into control animals; we have neglected to consider this point because in practice we have used, as the basis for the control of the value of a live vaccine, the resistance to a test whose severity left no room for any doubt and because, under these conditions, we had grounds to assume that the efficacy of a vaccine in man would be in direct proportion to that of a vaccine administered to the guinea pig which was said to be refractory to all immunization by means of the usual vaccines.

Hence, for us, TI (total immunity) attests that the

surviving animal has exhibited only an insignificant local reaction without modification of its habitual physiological behavior; PI (partial immunity) applies to the animal which survives* after showing a strong reaction in the form of an inflammatory lesion with adenopathy -- which may or may not be abscessed -- regressing in 3-4 weeks, the general state of health of the animal being affected during the first 6 to 10 days; AI (absence or disappearance of immunity) implies the death of the animal within a period of time and with symptoms that are comparable to those of the control guinea pigs.

On the basis of these data, our attention was retained particularly by one of the attenuated strains, strain EV, isolated in 1926 from a human bubo, which after five years of subculture on nutritive agar possessed the properties which we expected of a virus-vaccine to a maximum extent. It is on this sole strain that all our investigations were subsequently carried out, which made it possible for us to perform a large number of experiments on many animals which could be observed, if necessary, for as long as two years. Our collaborator J. Robic was thereafter associated with this study, and made a major contribution in the area of the application of vaccination to human immunization.

Characteristics of the EV Strain

Until 1934 we refrained from mentioning our researches outside the annual reports of the Pasteur Institute of Tananarive, whose publication, in a limited number of copies, started only in 1931**. Let us quote the essential passages which appeared in the 1933 report relating to the EV strain: 1) The strain is avirulent for the guinea pig (415 animals) when inoculated subcutaneously, even when the dose is as large as a whole agar culture tube. The rabbit, as well as the sheep and the calf, support large doses of this strain when applied intravenously. Doses of several billions of germs applied intraperitoneally in rabbits and guinea pigs are fatal to about 20-30 percent of the animals in 2-3 days; the animals die of

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*Subject to a pulmonary complication which sometimes causes the death of the guinea pig after 10 or 20 days, and whose belated appearance is no less a proof of the persistence of a certain degree of immunity.

We shall return to this fact later in this paper, because of the theoretical interest of this observation.

**In 1937 this publication became the *Archives de l'Institut Pasteur de Tananarive* (Archives of the Pasteur Institute of Tananarive), later *de Madagascar* (of Madagascar), brought out in the form of an annual brochure in which the various aspects of the work relating to plague, carried out on the large island were discussed in a long chapter.

peritonitis with septicemia. The peritoneal liquid may then be found to be virulent while the germ obtained by hemoculture is not. Hence, the EV strain cannot be considered as attenuated and fixed, but only weakened in its virulence (in the sense given to this term by M. Nicolle), since it is possible to restitute a part of its virulence by an experimental device.

2) This strain remains toxic, and probably owes its immunizing power to the persistence of this characteristic. White mice and rats whose sensitivity to the plague toxin is known (the guinea pig resists this toxin much better) support only weak doses of this strain, but we were never able to demonstrate the existence of the inoculated germ when we implanted their ground viscera into other rats or mice.

3) The immunity conferred on the guinea pig by a single inoculation (1 ml of suspension of a culture of an agar tube in 20 ml of physiological saline) is still apparent after nine months. After this time, and for certain animals after one year, the virulent test leads to the development of a carbuncle with adenopathy, lesions which heal within four weeks without any sequels. The immunity is also manifest vis-a-vis the infection transmitted in the natural manner, i.e., after being bitten by a plague-carrying flea (*Xenopsylla cheopis*) emerging from a dying rat. Two guinea pigs, one of which had been vaccinated three months before, were placed into contact with this flea. Only the control animal died of acute plague after six days; the vaccinated animal, sacrificed 20 days later, exhibited no lesion. In 1932 we reported that the immunity which was already apparent on the fifth day became total eight days after the vaccinations.

4) All controls -- 87 in all -- of our experiments died of septicemic plague, without exception, between the fourth and seventh day.

5) The best way of introducing the vaccine is the subcutaneous route, but solid immunities have also been obtained by the cutaneous route (scarifications), by the oral route, and by the ocular route (instillations).

6) The vaccine is more effective when it is used immediately after its preparation. After 15 days a decrease in its activity is noted.

7) One-hundred-and-twenty-eight vaccinated and nontested guinea pigs, sacrificed at intervals of six days to 13 months, exhibited no plague lesion. By hemoculture, by implantation of the homogenates of organs in the peritoneum or under the skin of guinea pigs or mice, we have never recovered a live bacillus. Hence, the EV virus-vaccine is incapable of creating bacillus carriers. Identical observations were made in the case of vaccinated and subsequently tested guinea pigs (42 animals) which were sacrificed 20 days to 10 months after the virulent inoculation.

8) It is possible to protect the guinea pig against pneumonic plague of the primary type produced by the direct introduction of virulent bacilli into the trachea by a subcutaneous inoculation of virus-vaccine EV.

9) Strain EV is inoffensive for man when administered in the form of a subcutaneous injection in doses of 500 million to one billion germs. We ourselves have supported these doses without damage on two occasions, and J. Robic on three occasions. These doses may apparently be increased without danger if we can judge by the experiment carried out on himself by our colleague P. Petrade (who subsequently rendered the most valuable assistance in the organization and execution of the vaccination programs): exhibiting an audacious confidence in the vaccine -- we would never have allowed him to undertake this test if he had consulted us about it before -- he inoculated himself with four billion EV bacilli; he developed a high fever for 48 hours and a local tumefaction which regressed in 25 days, without the least apparent ganglionic reaction and without his general state of health being affected.

10) The first human vaccinations were carried out 15 months ago. [The article quoted from was published in 1933.] The developments during this year permit us to state that they are absolutely innocuous; we believe that the immunity conferred by the EV virus-vaccine is superior to that provided by the usual vaccines.

First Applications to Human Vaccination

The responsibility for the first inoculations of the EV virus-vaccine in humans was assumed by our friend Robic who took it upon himself to carry them out without informing us during one of our stays in France. We have only recently, in a communication to the Society of Exotic Pathology [43], lifted the veil which had shrouded the circumstances, previously known only to a few initiated persons, which had caused our collaborator to undertake these experiments which we would never have permitted him to carry out, in our absence. Five lepers, kept under strict surveillance, were inoculated in August 1932 with 250 million germs each: the local and general reactions were insignificant. Four months later, seven other lepers received an identical dose at the same time that Robic carried out the test on himself; there was no incident. In January 1933 it

was the turn of 90 other lepers* and three service assistants who offered themselves as volunteers. Finally, 1600 vaccinations were performed between January and March 1933 in an infected environment, after authorization given by Governor-General Leon Cayla and by the Director of Public-Health Services, who had been confidentially informed about the situation in certain villages where plague was raging intensely and where, although there was a risk in employing a live vaccine, this risk was not to be considered any more serious than the danger which threatened the inhabitants of this sector. These inhabitants, thrown into a panic by a murine epizootic, abandoned their dwellings and tried to find refuge in hastily erected huts. Moreover, only volunteers were injected, but there were many volunteers and some had to be refused. Whether or not this was a coincidence, the result of these 1600 vaccinations was very encouraging: while plague continued to rage in villages adjacent to the sector in which the experiment was being carried out, there was only a single case of bubonic plague in the vaccinated sector, in one of the vaccinated children who, moreover, was cured without treatment despite the fact that the germ isolated from him was highly virulent for the guinea pig.

Five months passed during which all persons were checked. Nothing abnormal was found in them and, in particular, there was not a single case of pneumonic plague, while there were several such cases among the non-vaccinated subjects. We shall see later on why we stress this absence of pneumonic plague in persons who were only insufficiently protected by vaccination.

The objective aimed at by Robic in this undertaking favored by a combination of circumstances was, above all, to confirm the innocuousness of the new vaccine.

During our stay in Paris, we were able to carry out, at the Pasteur Institute, under the supervision of our teacher

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*The lepers were chosen by Robic because of the ease with which they could be supervised in an establishment isolated from all population centers where the highest degree of privacy was ensured and which, moreover, was situated in a sector devoic of plague. However, as the years went by, it became apparent that the lepers had acquired, in the advanced stage of the disease, an appreciable immunity against plague. Their reaction to the EV virus-vaccine could only be banal and less pronounced than that of normal individuals. See in this connection: G. Girard, "Behavior of Leprous Rats (Stefansky's Disease) Toward Experimental Plague Infection," *C. R. Soc. Biol.* (Proceedings of the Biological Society), 1951, Vol. 145, 1627-1630, and G. Girard, "Are Lepers Refractory to Plague?" *Bull. Acad. Nat. Med.* (Bulletin of the National Academy of Medicine) 1952, Vol. 136, 80-83.

E. Dujardin-Beaumetz, experiments similar to those which we had performed in Tananarive on the guinea pig; the results of these tests were in line with what we had expected.

The secret of the operations carried out by Robic in Madagascar could not be kept any longer. We addressed a confidential note to M. Roux describing the state of our studies and the institution of the practical measures to which these studies had led, without omitting the gravity of the situation created by plague in the large island. We knew in effect that M. Roux, a great friend and confidant of Yersin, had followed the latter's work with unflagging attention ever since his memorable discovery, and was well aware of the prospects of a vaccination with attenuated live bacilli which were opened up by his collaborator's tests. We have never failed to stress that Yersin, assisted by Carré, was the first person to have carried out experiments in this direction, and even inoculated himself, without great damage, with an attenuated plague bacillus which was still lethal to 20-30 percent of the rats, but which firmly immunized those which survived this vaccination [151]. By contrast, the monkey supported this virus-vaccine without incident; this vaccine protected it just as solidly as it protected the rat, and since the behavior of the monkey was, for Yersin, closer to that of man than the behavior of the rat, he deduced from this fact that man was capable of benefiting from this mode of immunization. However, Yersin took care to conclude his communication with the following recommendation, which we kept in mind from the very beginning and which was to guide our behavior: "It is always a serious matter to inoculate man with a bacillus which, even if it is somewhat attenuated, may cause accidents in certain cases. Hence, this method should be applied only with the greatest care, by surrounding oneself with all the necessary guarantees." Seventy years before Yersin's discovery, five out of the six persons who had been inoculated, in Egypt, with pus taken from buboes, with a view to vaccination, died [74].

Thus, we had the right to wonder whether M. Roux had not been inculcated by his friend with some of this precaution, which would then make it necessary for us to abandon the undertaking which was begun without his knowing about it. It is on his and only his opinion that the continuation or interruption of the vaccination in Madagascar with the EV virus-vaccine would depend.

"The documents* which you have communicated to me seem

*The photocopy of this letter (an extract from which is given above) remained unpublished until 1959; it is contained in our communication to the *Societe de Path. Exot.* [43]. The original has been sent to the Pasteur Museum, where it is preserved.

to justify the tests which you have undertaken in Madagascar. By continuing to carry out your studies under your present conditions, you and your collaborator will know promptly what the potentials of the EV strain are as an antiplague vaccine. It would be useful to collect the greatest possible number of virulent plague bacilli of various origin, to maintain them by subculture and to seek out among these strains -- after they have lost their virulence -- those which constitute good antigens."

M. Roux's letter which arrived on the eve of our departure from Marseille, and from which we cite these lines, dispelled our qualms; it led us to deliberately extend the application of the new vaccine, in view of the fact that at that time no other preventive method could be counted on.

Thirteen thousand new vaccinations were carried out between September 1933 and January 1934 in three sectors which were still in the throes of an epidemic; the documents collected afterwards all agree in that the vaccination was of incontestable value. We allowed the six months of the southern winter to pass, since that period is characterized by an annual regression of plague as well as by the recrudescence of lung diseases among the Malagasy; our aim was to gather a great deal of evidence on the innocuousness of the virus-vaccine. In June 1934 in a communication to the Academy of Medicine we gave an overall summary of our experimental studies and their first applications [44].

During the 1934-35 epidemic "season," i.e., from September to March, the project involved the district of Ambatolampy, where 46,000 of the 106,000 inhabitants were vaccinated. The operations carried out under our direction with the assistance of Drs. Estrade and Milliau, who maintained the most rigorous organization, execution and control possible in the midst of this population scattered over more than 1000 hamlets, definitely confirmed the value of the EV virus-vaccine. A systematic plague detection was carried out, regardless of the cause of death claimed; organ smears and samples of pulmonary and hepatic serosities were taken in the case of all bodies for inoculation into the guinea pig. The plague mortality was reduced by two thirds in the case of the vaccinated group compared with the controls, and the general mortality, by almost 50 percent. Furthermore, not a single case of pneumonic plague was recorded among the vaccinated persons, whereas there were 17 such cases among the controls. We particularly stress the fact that a general mortality rate -- excluding deaths from plague -- that was equal or higher in the vaccinated subjects than in the controls would have cast doubt on the innocuousness of the vaccine. In the report prepared after this vaccination program, whose essential data were published [45, 46], we wrote, notably, the following:

"Some cases of plague cannot be confirmed bacteriologically after death by the microscopic examination of the smear, the culture or the inoculation: the reason may be that the samples were incorrectly taken, the buboes were unrecognized and the body was in the process of putrefaction -- gaps which are especially frequent in the brush, independently of the frauds which one must always think of in a native environment. The official figure given for the number of plague cases recorded in the infected sectors of the Emyrne does not in itself take into account the bell-shaped curve of the general mortality rate during the epidemic period: this has been the case for years."

The administrative authorities were struck by this decrease in the overall number of deaths in the group of vaccinated persons, but the Malagasy themselves were no less surprised. It is partly to this latter fact that we attribute their subsequent favorable acceptance of the new vaccine.

The Massive Vaccination Programs. Their Results.

The Pasteur Institute of Tananarive was organized in 1935 in order to be in a position to manufacture vaccine for the immunization of the populations exposed to the infection which slowly gained a foothold over the entire Plateux region, where more than 1,500,000 persons were living. Provisions -- which we shall not go into -- were made in order that the operations could be carried out rapidly, and they were always strictly controlled by a trusted personnel. We shall limit ourselves to recalling that the annual vaccination campaigns (which comprised several revaccinations, since we know that in animals the immunity fades out after about ten months) were begun at the start of the epidemic season, i.e., in September, and terminated during the next two months. No vaccination was carried out outside this period.

In Table I the "epidemic year" extends from 1 May to 30 April; the other two columns list the number of inoculations with the vaccine and the number of plague cases, respectively.

Epidemic Year	EV Vaccinations	Plague Morbidity
1933-1934	12,000	3,493
1934-1935	46,000	3,605
1935-1936	714,244	3,033
1936-1937	633,000	1,376
1937-1938	796,956	596
1938-1939	400,000	628
1939-1940	176,718	912
1940-1941	813,000	449
1941-1942	621,000	185

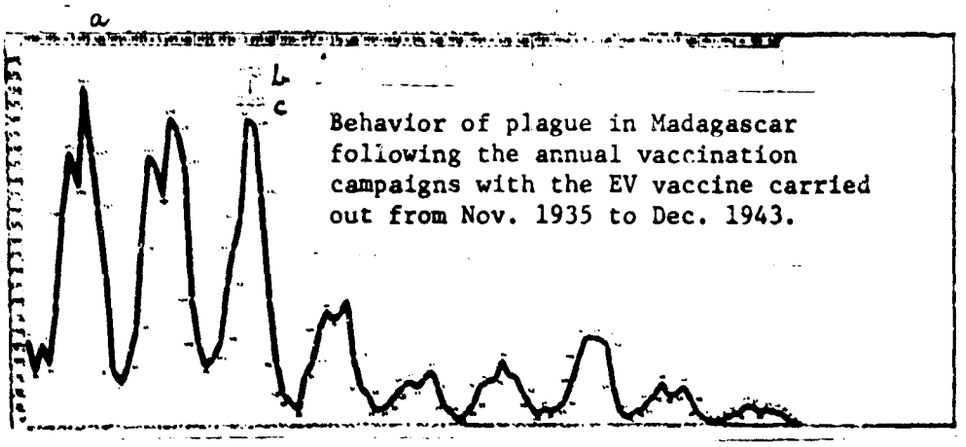
The diagram, whose component elements purposely terminated in 1942, shows the correlation between the vaccinations and the drop in plague morbidity.

Later in this paper we shall discuss the evolution of the situation between 1942 and 1959.

Comments. Interpretation of the Results

No matter how suggestive the sharp decline in the plague curve in Madagascar may appear between 1936 and 1942, can we attribute it solely to the new vaccine? By themselves the figures speak eloquently, but comparable results can be found if we examine certain statistics published in India and elsewhere during this pandemia, which has not prevented certain individuals from endlessly advocating the value of vaccination and others from expressing a harsh criticism of vaccination; the latter were persons who underwent vaccination because they were required to do so, and who only noticed its lack of success. In fact, these statistics were always drawn up on dubious foundations, as was mentioned by Otten who, independently of us, developed a virus-vaccine in Java and carried out massive vaccinations with incontestable success. We shall return to Otten's work when we compare the characteristics of the Javanese strain (Tjiwidej) with those of the Malagasy EV strain. However, this author deserves much credit for having shown by means of an experiment which is irreproachable both with regard to its conception and its execution and which was carried out on 35,000 persons, that his live vaccine was superior to the killed vaccine -- Haffkine's lymph -- which he had received from the Bombay Institute or which he had prepared himself. Otten felt that one cannot correctly interpret a comparison between vaccinated and control groups unless the operations had been carried out according to the so-called alternate method which implies the immunization of one half of the inhabitants of each dwelling. In this way one obtains comparable controls, because all persons are exposed to the same risk of contamination [107]. On the basis of this fact he was able to establish that if the killed vaccine reduced the number of plague cases by 50 percent compared with the cases occurring in the controls, this reduction would attain 80 percent with the virus-vaccine. This experiment which preceded the collective vaccinations in Java is, to our knowledge, the only one which has ever been carried out. Such an undertaking requires, in effect, a set of favorable conditions including the assistance or at least an absence of reticence on the part of the populations on which the experiment is carried out. This undertaking would have been impossible under this "experimental" aspect at Madagascar where the measures of antiplague prophylaxis, and especially vaccination, had for ten years been fighting an increasingly

uphill battle; they were considered by the natives as a form of harassment because, apparently, that had not stopped the spreading of plague, the number of whose victims grew from year to year. However, the analogies between the observations made at Java and at Madagascar only affirmed the encouraging impressions which we have derived from the "pilot" immunization campaign carried out in the Ambatolampy sector in 1934-1935.



Behavior of plague in Madagascar following the annual vaccination campaigns with the EV vaccine carried out from Nov. 1935 to Dec. 1943.

Fig. 2. Monthly Number of Plague Cases as a Function of the Seasonal Epidemic Cycle, i.e., from 1 May to 30 April of the Following Year.
a -- cases. b -- campaign. c -- vaccination.

The first mass vaccination (1935-1936) was off to a rather belated start, at a time when the situation was so bad that the figure of 3,605 cases of the previous year was about to be exceeded. Nevertheless, this figure was reduced by 600. Hence, as we mentioned above, it was decided to become organized in order that the following campaigns could be carried out between September and November, and so that we would be ready to intervene at any moment in limited sectors in order to eradicate foci of a particularly severe character. We have reported with Robic [47] that on many occasions we have had proof of the immediately effectiveness of the vaccination, in that the evolution of an epidemic of bubonic plague was clearly arrested. From this document let us cite only the following two significant episodes.

1. In a village of 74 inhabitants where seven fatal cases of bubonic plague resulting from a murine epizootic had just occurred, we found a built-up area that had been completely evacuated on the day before our arrival. The inhabitants took refuge in straw huts hastily erected on an adjacent hill. With the exception of six contacts left for observation

at the quarantine station, all persons were vaccinated. Ten days after this vaccination we advised all inhabitants to return to their cabins which had not been disinfected nor had the rats been killed: the village was left exactly as it had been at the time of its evacuation. Only a single case of plague was observed among the six isolated, non-vaccinated persons; afterward not a single case was reported in the village. Now, on the basis of what we know of the role played by the murine fleas (*A. cheopsis*) moving freely in the dust of cabins where human plague manifests itself after the death of the rats, it is unlikely that the contamination of one of the six non-vaccinated persons was due to an accident, while the 61 vaccinated persons who ran the same risks were spared.

The village of Mahatsinjo is irregularly visited by plague. In March 1937, six cases of bubonic plague were observed in a hotel frequented by the Malagasy. After a one-year interval (April 1938) the infection broke out again in an establishment very close to the preceding one; in a single week the owner of the hotel, his wife, his grandson and a neighbor died of bubonic plague. In the presence of one of us the contaminated houses were closed after the most thorough disinfection possible; the operation was completed by careful sweeping and by the incineration of the mats as well as of the dust collected during the sweeping, since we had reason to believe that this dust contained infectious fleas. We then vaccinated the population living nearby, a total of 196 persons. The houses were re-opened after 15 days. Numerous persons entered the rooms in which the hotelkeeper's furniture was distributed. The next week three cases of plague were reported; they occurred in the only three non-vaccinated persons who took part in this liquidation. Does not this episode have the value of an experiment and does it not demonstrate the ineffectiveness of disinfection measures applied at that time to the native cabins, as well as the reality of the protection conferred by the EV virus-vaccine?

Nevertheless, it did not escape us that certain fortunate coincidences governed by the secret workings of the "epidemic spirit" could be adduced -- and there was no lack of such conjectures, even on our own part -- in order to find an explanation for this strong regression of human plague in Madagascar, in this way reducing the importance of the new vaccination [48]. In numerous countries plague had been in a state of marked decline for many years without our being able to attribute this decline -- except in the case of the Dutch East Indies -- to the application of an original method of vaccinal prophylaxis. To solve this question we made use of supplementary evidence collected during the years 1939 and 1940 and published two years later [49]. First of all, it should be kept in mind that, in the central region of Madagascar, plague

had made its first appearance only in 1921, much later than in the countries of Africa and Asia, to which we have referred above. Then, the epidemiological conditions, unchanged for 15 years, differed considerably from those of the other endemic territories such as Morocco, for example, in connection with which Remlinger wrote [119]: "When plague strikes a new country, it has a marked tendency to carry out its up and down cycle in 3 or 4 years; hence, the decline in the mortality rate from 90 percent in 1909 to 20 percent in 1912 seems to herald its end in the near future," However, in Madagascar the situation was quite different, and we had to rely on the evidence that plague was constantly spreading, without abandoning any of its initial foci, between 1921 and 1936, the year in which the general vaccination with the EV virus-vaccine was begun. Finally, by contrast to what had been observed in India after the severe epidemics of the beginning of the century when the marked resistance of the rat to plague infection was considered in certain sectors as an important factor in the arrest of the epidemics, the receptivity of the domestic rat (*R. r. alexandrinus*) to the infection remained very high: in 1932 it was 85 percent, and during an experiment carried out in 1940 with four batches of 50 animals each it was 90 percent. Moreover, it is on the basis of observations of this type and also of others relating to the behavior of the virus reservoir that we have stressed how much the epidemiology of plague, as well as its physiognomy, may vary from one territory to the other, and even more so in various sectors of the same territory (which is the case of Madagascar) because of the predominant effect of climatic factors on the virus reservoir, the latter being represented primarily by the pestiferous fleas. However, quite apart from any theoretical discussions, the facts are there to bear out our belief in the value of vaccination from the point of view of the incidence of human plague during this period of 1937-1942. The sustained effort during three years of intensive immunization campaigns led us to limit the application of the vaccine in 1938-1939 to the most highly infected districts; with 400,000 vaccinations or re-vaccinations, the number of cases of plague remained essentially at the level of the preceding year, 628 vs. 596. For 1939-1940, the program still included the execution of 400,000 vaccinations but the perturbations due to the mobilization prevented the complete realization of this plan. The plague curve exhibited a peak with 912 cases, compared with 628 in the previous year. Hence, in 1940-1941 we resumed massive vaccinations: 813,000 persons were then vaccinated or re-vaccinated, and the plague morbidity again exhibited a decline: 450 cases compared with 912.

Finally, our point of view would be significantly strengthened if we took into consideration the special situation of the urban center of Tananarive during these five years.

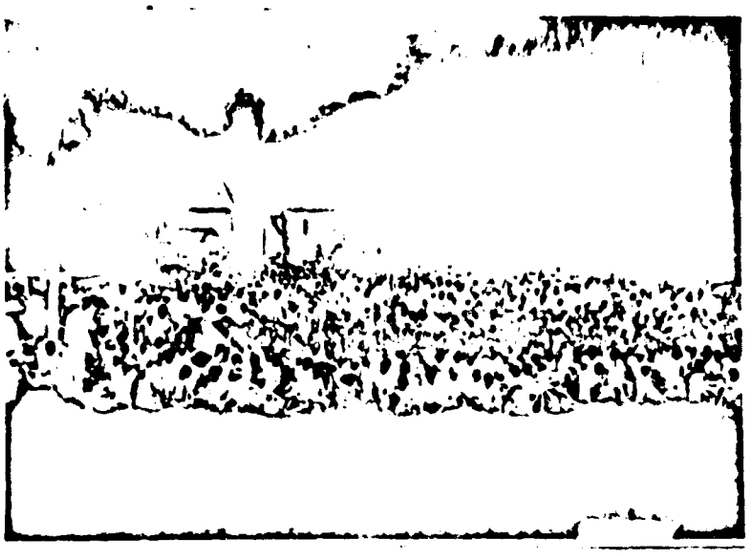


Fig. 3. Gathering of Malagasy for a Session of Antiplague Vaccination in the Brush. (Photo: Pasteur Institute, Tananarive).

If the regression of the plague had obeyed a natural law, then the center which was first affected by the epidemic should have been the first to manifest a regression. In addition, the public-health organization had been gradually perfected in this city of 100,000 persons where the post-mortem detection of the disease was systematically carried out, where the isolation of persons and the prevention of contacts was rigorously enforced, and where a service of deratization, disinfection and disinfestation was in operation with a specialized personnel. In the rural sectors -- as we have stated above -- these methods of causal prophylaxis were, for various reasons, more or less ineffective. Hence, in agreement with the directorate of the public-health services, we believed that we could suspend the vaccinations in Tananarive for at least one year after the two campaigns of 1937 and 1938. The morbidity dropped to such a low figure in the city (19 cases) that we were inclined to believe that plague would disappear from the capital. Actually this was not the case at all. In 1938-1939 there were 54 cases, in 1939-1940, 70 cases, and in 1940-41, 97 cases when there were still two months left before the end of the epidemic year. These figures are significant because they represent isolated cases and not manifestations of pneumonic plague which is spread exclusively by interpersonal contact, manifestations which sometimes

weigh heavily on the statistics but which do not, from the epidemiological point of view, have the importance of cases of bubonic plague which attest the persistence of an aggressive virus reservoir. However, when it was announced that we would resume the vaccinations at the end of 1939, the population of Tananarive showed no inclination whatever to submit to them, and the few thousand inoculations which were carried out could not notably influence the physiognomy of human plague in this large urban center. By contrast, in the rural areas the vaccinations were carried out actively and successfully; for the first time there were fewer victims in the rural districts than in the capital, considering the relative population figures. Does not this comparison give an additional proof of the efficacy of the virus-vaccine and the reduced importance of the heavy sacrifices made in the attempt to eradicate the virus reservoir in the urban sector at a time when this control was not yet based on a rational disinfestation by modern methods? In all objectivity -- as we wrote jointly with Robic [47] -- both epidemiology and clinical medicine came to our support in challenging the assumption of a spontaneous regression of plague which, in 1937, showed itself just as aggressive and lethal as in the preceding years, if we take into account the morbidity rates in non-vaccinated persons. Furthermore, in the cantons where practically all inhabitants -- 92-95 percent -- were vaccinated, the results were always impressive: they were less so where the number of vaccinated persons was less than 75 percent. There is not a single plague focus that had not been eradicated by vaccination alone, in the absence of any other measure.

To sum up, the EV virus-vaccine, which has never failed to give evidence of its great effectiveness in the laboratory, clearly demonstrated its immunizing effect after three intensive vaccination campaigns which involved a population of slightly more than one million persons, 800,000 of whom were vaccinated and many re-vaccinated. The plague morbidity was reduced by 80 percent. We could report that the vaccination by a live vaccine remained the fundamental element of the struggle against plague, the only one on which we could rely to ensure the protection of the rural populations of Madagascar, as long as it was impossible to effect radical changes in the conditions of native dwelling [49].

Evolution of the Situation (1942-1959) and the Vaccinations

After returning to France in 1940, we continued our research at the Pasteur Institute, while remaining in close contact with our colleagues in Tananarive. The person who now became responsible for the vaccination program was our successor J. Robic until December 1953 (except for 1947-1949 when

R. Favarel was in charge), and J. Courdurier and E. R. Brygoo since 1953.

After 1939 it was expedient to limit the operations to the most intensely threatened sectors. As is shown in Table I, the drop in the number of vaccinations to 176,718 in 1939-1940 unfortunately coincided with a marked increase in morbidity. The directorate of the Public-Health Services asked that the vaccination be stepped up again: for them this seemed to be the sole effective method of prevention. The results confirmed this impression because with 813,000 and then with 621,000 inoculations, the number of cases of plague dropped from 912 to 449, and then to 185*.

From then on the plague was controlled, and although the vaccinations have never been completely interrupted and remain indispensable under certain circumstances, we believe that it would be erroneous to overestimate their importance in the evolution of the epidemic situation shown in Table II.

Table II

Epidemic Year	EV Vaccinations	Plague Morbidity
1942-1943	417,000	241
1943-1944	360,315	185
1944-1945	93,000	199
1945-1946	164,000	197
1946-1947	63,531	312
1947-1948	415,000	278
1948-1949	400,000	158
1949-1950	12,000	109
1950-1951	0	241
1951-1952	80,000	157
1953-1954	438,000	97
1954-1955	536,146	14
1955-1956	799,907	25
1956-1957	881,158	38
1957-1958	756,126	62
1958-1959	83,000	25
1959-1960	0	23
1960-1961	0	20

*Vaccination was always carried out between 1940 and 1955 (J. Robic) in all European or native military personnel, who were subjected -- as everywhere in the armed services -- to a strict medical surveillance. There was not a single case reported among them since 1940, despite the operations which in 1942, and then between 1946 and 1947 during the repression of the rebellion, led large contingents to move to and be stationed in rural zones known to be endemo-epidemic foci.

In effect it is necessary to take into consideration certain new facts which may be attributed to the perturbations caused by the hostilities in 1942 and especially to the 1947 rebellion whose sequels have entailed great population movements within and outside the infected regions. Should we consider these movements to be the reason for the development of plague foci in sectors which up to then -- and perhaps because of the absence of reliable data -- had been considered free from the germ? Whatever the case may be, the 1948 and 1949 reports underline this new epidemiological factor, in relationship to murine infection, and the vaccinal prophylaxis involved several thousand inhabitants dispersed in these distant cantons. However, for three years after 1949 intensive domestic disinfestation campaigns were carried out by means of DDT, which were undertaken in the principal centers both for the eradication of malaria and of plague, with brilliant result. Mercier and Razafindrakoto who had directed this campaign in the district of Tananarive-ville were able to write in 1953 [102]: "As for plague, while cases occurred at an accelerated rate since 1921, and while the city was never free from the disease for more than three consecutive months, we have not had a single case between 10 August 1949 and 1 June 1952, a period of 34 months." And they added: "For one year the pulicidal index remained at about 0.15 per rat (15 fleas per 100 rats captured, instead of the previous average of 6 to 8 per rat). The *cheopis* index is around 0.04 (formerly it was always greater than 1, and most often 1.5 per rat), and the percentage of *X. cheopis* oscillates between 20 and 34 percent (previously it was between 50 and 70 percent of the total number of fleas captured)."

Now, during this period, not a single vaccination was performed in Tananarive, and it would be contrary to all evidence if we failed to attribute the absence of human and murine plague during these three months in the Malagasy capital to this systematic disinfestation. However, at the same time, the number of plague cases had more than doubled in the rural sectors, and one could wonder whether this was not due to the interruption of vaccinations. The Malagasy, especially, expressed this view openly, and I heard from Robic that certain persons went as far as to state that the reason for the interruption was that the EV strain had disappeared or at least lost its protective effects. Trusting this vaccine whose benefits they could appreciate for years on the basis of their own observations (because, better than we, they were able to make useful comparisons in their environment between the behavior of those persons who had been vaccinated and those who had not), this represented a psychological factor which the responsible authorities could not neglect, especially since a slight increase of the incidence of plague in 1952 made it necessary to resume the vaccinations. From 1954 to 1958, the number of vaccinations

carried out was quite high, resembling that of the first campaigns. Was there a proportional effect on plague morbidity? To be sure, the vaccination has reinforced the immunity of those who allowed themselves to be revaccinated, but the risks which these persons ran did not have a common denominator with those who had lived 15 years before. If we examined the figures, a brief analysis of the latter would reveal certain paradoxes for the period of 1955-1958. Nevertheless, in this irregularity of the morbidity which was without apparent relationship to the intensity of the vaccinal prophylaxis, one fact predominates: since 1953 the number of plague cases has, for the first time since 1921, dropped below 100, and is still below that figure.

However, there is another reason which has caused the Directorate of the Public-Health Services to resume vaccination, a reason communicated to us by our colleagues in Tananarive, and which is expressly mentioned in their 1958 report as follows: "One may be tempted to conclude that a regular decline of the plague has taken place, and that this disease is on the way toward extinction in Madagascar. This would be an erroneous and dangerous conclusion. Plague is still present, and is as active as before, but we are now witnessing an artificial regression of the number of cases due to the disobedience (whether or not intentional) of the prescriptions decreed for plague detection. Of the 194 examinations requested at the Central Plague Service in 1958, 185, or 93.3 percent, came from the province of Tananarive. In particular, we have not received a single sample from the provinces of Fianarantsoa and Tamatave where well-known endemic zones nevertheless exist. It is impossible to assume that in these regions not a single case of death had occurred in 1958 under conditions which would have called for an investigation of the deceased. Only one conclusion can be drawn: we do not have, at the present time, reliable data from any part of the country other than the province of Tananarive. This is the only province that is regularly checked. It is to be hoped that the artificial calm which has obtained as a result of the neglect of the regulations will not be broken by a rude awakening; it was not long ago that the efficacy of these regulations was confirmed."

How wise is this reflection of our colleagues, and how many examples we could cite, together with Robic, of the disastrous consequences which have resulted in the past from the nonobservance of the urgent prescriptions relating to the systematic plague detection in Madagascar [50]. Without this measure, which was never accepted without reluctance on the part of the Malagasy people [51], we would have remained completely in the dark regarding the actual incidence of human plague, and we would have seen the manifestations of epidemic pneumonic plague increase in number, which has in fact happened on several

occasions, attributable to a pulmonary complication in a patient with unrecognized bubonic plague. The last episode of this nature was reported to us by Brygoo and Gonon: this is a recent report because it dates from October 1957 [11]. It happened in a rather inaccessible region of the northeastern part of the island where no cases of plague had previously been reported; in a few days it had caused the death of 36 persons. Since the alert was sounded only after a great delay, our colleagues still found six persons with plague at the site; they were cured with streptomycin. The origin of these 42 cases of pneumonic plague was a sick person who had arrived from a village situated in a region which had been known as an eccentric focus of bubonic plague only since 1955.

These considerations, which at first sight seem to go beyond the scope of our topic, nevertheless belong there, because they show one of the aspects peculiar to plague in Madagascar since 1921, an aspect which we have treated at length elsewhere [52]. While plague is generally in a state of decline throughout the world, it continues to exist in Madagascar, and vigilance is still necessary in view of the threat of these manifestations of pneumonic plague, as we have just related; it will be necessary to be on the alert until the complete eradication of the virus reservoir, which can only be expected from nature, at least in the rural regions. The preceding example also proves that the virus has lost none of its aggressivity. As for the fluctuations exhibited by the statistics during the last six years, they are insignificant, and in the future they will be greatly subordinated to the incidence of pneumonic plague which is spread exclusively through interpersonal contagion; in the absence of adequate epidemiological indications it will be this incidence on which, in the final analysis, the distribution of vaccination in depth will depend.

Pneumonic Plague and Vaccination

When we speak of antiplague vaccination, we always mean bubonic plague. It has always been assumed that vaccines failed to protect man against primary pneumonic plague having an epidemic tendency, whose pathogenesis differs considerably from that of the bubonic form, and in whose case individual protective measures (wearing of a mask and goggles) were sufficient to prevent contagion. It is not paradoxical to maintain that it is easier to escape pneumonic plague than bubonic plague, provided that it is detected before the focus has had time to develop and that one has been able to isolate the first victim or victims. Today, the preventive administration of sulfonamides for a few days to all persons in contact with a patient with pneumonic plague has given ample proof of the efficacy of this measure. If we add that the patients treated in time with

fungal antibiotics (streptomycin in particular) get well in a few days, it will be agreed that epidemic pneumonic plague can no longer provoke in a developed country the panic which it had caused during the Middle Ages, and which took place, in more recent times, in Manchuria in 1911 where 50,000 victims perished in three months. It is under this aspect, which perhaps is less impressive due to the low population density, but which is identical in its process, that we have made our acquaintance with this fearful form of infection in the central region of Madagascar, and were able to show that this was intimately linked with the bubonic form, a pulmonary complication of which would give rise to the first case of primary pneumopathy responsible for an epidemic spread by interpersonal contact [52]. In view of this situation which is inherent in the nature of the climate characterizing this part of the territory, not only did we have to ask ourselves whether the EV virus-vaccine would afford protection against pneumonic plague, but also whether it would not contribe to its spreading!

Ever since 1899, experiments (Batzaroff, Vassal) have revealed that guinea pigs and rats which received vaccine or antiplague serum for protection purposes in a dose which was insufficient to make them completely refractory to the disease, belatedly died when subjected to a virulence test, due to secondary bronchial pneumonia, with the presence of an enormous quantity of plague bacilli in the lungs and only a few germs in the liver and the spleen, contrary to what is observed in bubonic plague with its terminal septicemic phase. In other words, this secondary pneumopathy is readily produced if the animal's organism exhibits a certain resistance to the invading microbe. In his vaccination tests with live germs carried out in Java, Otten also mentions the death of incompletely protected guinea pigs as a sequel of secondary pneumonia. We have confirmed this finding in every respect in laboratory rodents, notably in guinea pigs which had been vaccinated many months previously, and whose immunity had faded. A few years ago we were able to see, on autopsy of two mice which had died under these conditions, how this process takes place; we noted an adenitis in the process of absorption on the paw where the virulent inoculation had been applied three weeks earlier; a lymphatico-sanguine cord on the same side, ending in the armpit where a new bubo was developing; then, through anatomic connection, invasion of the lung covered with lesions of the pseudotuberculous type in which the specific bacillus abounded; the latter was very rare in the spleen and the liver, which were apparently normal.

In truth, the practice of vaccination and serotherapy, which has been carried out since 1895 on millions of individuals has nowhere allowed making such observations, and the question never even arose in the case of man, as it had in the case of animals, as to the development of pulmonary complications during

attacks of bubonic plague whose evolution would be prolonged by means of a partial vaccinal immunization. It will be agreed that the circumstances have hardly lent themselves to such observations in countries such as India where pneumonic plague has always been exceptional in its epidemic manifestations. This was not the case in Madagascar, and if man responded like the animal, we would have risked the development, in the case of incomplete protection -- vaccination by the EV virus-vaccine, too, had its failures -- of this complication which has such fearful epidemiological consequences. This problem was all the more serious since the substitution of a live vaccine for a killed vaccine would not have failed to raise the question of the possible increase of the virulence of the inoculated virus-vaccine, which is the cause of these pulmonary manifestations. We have waited ten years before answering this question in the negative in an article from which we quote the following passage [53]: "The caution with which Robic and ourself have carried out the first vaccinations between 1932 and 1934 was in part justified by the scruples resulting from our experimental findings in a country where the conditions were so favorable to the spreading of pneumonic plague. As we were limited to carrying out the vaccination in an epidemic environment, an increase of the number of cases of pneumonic plague, even if due to accidental factors, would certainly have interfered with the progress of the new vaccination." Luckily there was no such increase; moreover, we were able to state later that, contrary to Otten's opinion, the EV vaccine was not ineffective in providing protection against pneumonic plague; the latter author, considering that his live "Tjiwidej" vaccine does not provide immunization against this form of plague, only included bubonic plague in his statistics, and deliberately set the pulmonary cases apart*, whereas in Madagascar they were included in our overall statistics on plague morbidity and mortality. Thus, if we refer to the tables of the above-mentioned articles, we note that the number of pulmonary forms has decreased between 1933 and 1941 under the influence of the EV vaccination, parallel to and in the same proportions as the total number of plague cases. The percentage of pulmonary cases compared with all patients with plague has remained at an average figure of 30 percent (maximum 32 percent, minimum 26 percent). As for the percentage of cases of pneumonic plague among vaccinated persons (to the extent that one may believe the statements made by certain carriers of vaccination cards), it

*While the manifestations of pneumonic plague were not as widespread in Java as in Madagascar, they were not exceptional in Java where some of the climatic conditions at a high altitude are similar to those of the high plateaux of Madagascar.

corresponds to this same figure of 30 percent. After the execution of four million inoculations, this question was definitely settled. A lesson was learned from these campaigns: first of all that there is a striking contrast between man and rodents from the point of view of the modifications brought about by the vaccination or sero-prophylaxis, respectively, in the evolution of the infection when the protection is only partial and, second, that while animal experimentation is an indispensable part of our study and offers many data, it is no substitute for human experimentation, when the maximum guarantee of safety is assured, to use the expression of Charles Nicolle: it is human experimentation alone that enables us to make judgments and draw conclusions.

We mentioned at the beginning of this article that it was possible for us to immunize guinea pigs against primary pneumopathy provoked by the introduction of virulent bacilli directly into the trachea. Robic's experiments on macacoes, lemurs which are native to the Malagasy fauna (which does not include any monkeys) and which are very sensitive to experimental plague, have shown that this animal -- like the guinea pig -- was protected by the EV virus-vaccine not only against the bubonic plague but also -- at least in the case of two specimens *Lemur mongoz* and an *Epilemur* -- against a pneumonic plague which is easily determined by the deposition, on the surface of the nasal mucosa, of a material that is virulent and quickly fatal for the controls [121]. It is in view of these facts that we are inclined not to subscribe to Otten's opinion regarding the impossibility of conferring some degree of protection in man against pneumonic plague.

Antiplague Serum and EV Strain

Shortly after the start of vaccinations, it seemed indicated to employ the EV virus-vaccine in the preparation of the antiplague serum which, at that time, and until the advent of the sulfonamides, was the sole medication available for bubonic plague. After a few experiments carried out on rabbits inoculated intravenously, Robic proceeded to the hyperimmunization of a horse at the Pasteur Institute in Paris. The preventive and curative value of this serum was confirmed in the mouse and the guinea pig. After that, the horses producing serum were prepared exclusively by intravenous inoculation of suspensions of EV virus-vaccine at increasing doses. The collected serum was found to be greatly superior in the treatment of bubonic plague to that of horses which had been treated with killed antigens, the latter having been substituted in several serotherapeutic institutes to live and virulent antigens due to the danger presented by their handling to both man and horse. There has always been a major waste in the case of horses hyperimmunized against plague, not because this animal is receptive to the

infection, but because of the immediate protein shock -- often a mortal one -- caused in the horse by the intravenous inoculation of antigen. Now, we can see that the sudden death of a horse which has just received billions of virulent plague bacilli in its circulatory system raises an important problem of safety to the knacker who deals with the animal's hide. We know, on the other hand, that the serum obtained after the subcutaneous introduction of live or killed plague antigens has only a mediocre therapeutic value.

In the USSR, Korobkhova [86] uses the EV strain as well as other avirulent strains in the rabbit and sheep, administering it in the form of an intravenous inoculation and obtains a serum provided with a high protective power in the mouse.

However, for the last 15 years, serotherapy has been relegated to the background and practically abandoned in view of the high effectiveness of antibiotic therapy. Nevertheless, the serum remains indicated as a substance to be combined with chemical -- essentially antibiotic -- therapy in certain hypertoxic forms of plague, as has been pointed out by R. Devignat (personal communication which we have greatly valued) and as we ourselves have seen in Madagascar [54].

For the experiments, for the obtainment of sera having a high agglutinating and precipitating power, and for studies relating to the active constituents of antiplague serum, the EV virus-vaccine represents an ideal antigenic material.

First Experimental Studies Carried Out Abroad on the EV Strain

Without exceeding the scope of the first part of this paper, we shall summarize the studies which have been carried out abroad between 1935 and 1940 in the direction which we have followed for the study of the experimental protective power of the EV virus-vaccine.

Our initial publications, as well as those of Otten, aroused great interest abroad, and we were asked by the directors of several government laboratories to provide them with the EV strain.

Otten, at the Pasteur Institute of Bandung (Java), confirmed the high immunizing power of this strain in the guinea pig, a power that is superior to that of his "Tijwidej" strain [107]. At Johannesburg, after recognizing the effectiveness of the EV virus-vaccine in the rat, Pirie and Grasset substituted this antigen for the killed cultures for the hyperimmunization of horses from which the serum was to be prepared [112]. At Buenos Aires, an important study by Anchezar, carried out under the direction of Sordelli [3], and another by the same author and Savino [131], completely confirmed our conclusions on the immunizing value of the live EV vaccine, and on the role of the

vaccinal reactions in the guinea pig in the genesis of this immunity, reactions to which we shall return later on.

Vincke and Janssens [145] reported that in doses of 1-2 billion germs the EV virus-vaccine confers immunity on 97 percent of guinea pigs against the inoculation of a standard lethal dose of a highly virulent microorganism such as the plague strains isolated in the Lake Albert focus; a killed vaccine (Haffkine's lymph) protects onl, 12 percent of the guinea pigs subjected to this test. They concluded from their experiments that the advantage of the vaccination by live organisms appeared to justify its use in man, inasmuch as several thousand inoculations have been carried out since 1938 without any adverse effect.

In personal communications, our colleagues at the Pasteur Institute of Dakar informed us of the results of their investigation. These results were satisfactory in the tests carried out in 1933 by Advier on monkeys and guinea pigs, mediocre or zero in tests carried out by Maurice Mathis, and again favorable in the experiments of Arquie who was provided with a new subculture.

In South America, relative or absolute failures were obtained in Lima and Santiago with cultures prepared from the same sample which had given the above-mentioned successful results in Buenos Aires (oral communication from Dr. Suarez to Robic). We then had a feeling that modifications which are a priori unpredictable are capable of intervening in the behavior of the strain, and one of our major concerns was to determine, by repeated controls, the causes of this degradation of the antigenic value and to find means for remedying it, which will lead us to a detailed definition of the characteristics of the EV strain and by extension of those of other strains of *P. pestis* designed for use as virus-vaccines. Although this problem presents itself to everyone responsible for the manufacture of all live vaccines, as far as our vaccine is concerned this problem was to be of very great interest, as we shall see in a special chapter which will be devoted to it.

The study of the EV strain was carried out most thoroughly in the USSR; it had been started in 1936 by Korobkhova, and was the starting point of a series of articles in which several researchers participated. These papers were collected in 1956 in a monograph which, at the present time, represents a valuable reference work on antiplague vaccination by live germs [87]. First of all Korobkhova, Bessonova and Tumanski confirmed the innocuousness of strain EV, its high protective power compared with that conferred by killed vaccines and other strains devoid of virulence. They, like ourselves, recognized that the strain possesses a certain degree of virulence which is not increased after inoculation in a guinea pig, but which accounts for its immunizing power, as indicated by the mode of reaction

of the animal. However, these researchers soon oriented their studies toward immunization against pneumonic plague, because they were aware of the fact that the usual vaccines are powerless to provide protection against this disease. They also knew the extent to which Manchuria, Mongolia and the Transbaikal area had been afflicted with pneumonic plague during the previous 50 years and how it still threatens the regions contiguous with the endemic focus of Central Asia from where the epidemics originate. In 1939, Korobkhova and Krainova [88] reported that they can produce in the guinea pig a primary pneumonic plague in 100 percent of the animals through inhalation of very virulent plague bacilli administered in the form of aerosols which are capable of penetrating all the way into the lungs; the guinea pigs treated in this manner died within 3 to 5 days as a result of specific pulmonary lesions. As for their immunization tests, we can do no better than to quote verbatim the summary given by these authors in French:

a) "The guinea pigs are protected in an absolute manner against primary pneumonic plague with the EV vaccine administered by subcutaneous inoculation, nasal instillation or through combination of these two modes of vaccination."

b) "After a single injection of a thick EV vaccinal emulsion all guinea pigs are protected against primary pneumonic plague produced by inhalation. The guinea pigs immunized by subcutaneous inoculation, followed (three days later) by a nasal instillation, are better protected than those which receive three nasal instillations."

c) "The duration of the conferred immunity is a function of the antigenic properties of the vaccine and of the method of vaccination."

These results support those which we have discussed in our 1933 report (referred to above) and in our subsequent publications [38].

The EV Virus-Vaccine in Man Outside Madagascar

In the Union of South Africa. Grasset immunized all troops from that country which were destined to become part of the expeditionary force in Madagascar between 1941 and 1944. This contingent was composed of a total of 1212 persons, including 401 Europeans, 615 blacks and 196 Euroafricans. In the meanwhile our colleague has studied several locally isolated strains of *P. pestis* and one of them -- strain K/120 -- which was considered avirulent, was found to be highly protective for the guinea pig and the rat. He employed this strain for the vaccination of about 40,000 persons during several epidemic manifestations which broke out in South Africa, the Basutoland and South-West Africa. As in the case of the EV vaccine, the immunization required only a single inoculation with 1 billion

germs. The reactions were mild, and the vaccination was readily accepted. The epidemiological and immunological analysis of 15 cases of plague which occurred in the vaccinated group, taking into consideration the date of immunization, the date of the infectious contact and the evolution of the disease, made the author conclude that the protection is already apparent after five days and is fully attained on the tenth day [76].

The reason why we believe it necessary to report these tests carried out with a strain different from ours is that they were inspired by those carried out in Madagascar in which our late lamented friend and colleague Grasset was greatly interested, and who has called the attention of H. Pirie, chief of the Plague Service at the Johannesburg Biological Institute, to these tests. In addition, his work represents an important contribution to the antiplague vaccination of humans by means of a live vaccine.

In the former Belgian Congo nearly 500,000 vaccinations were carried out with the EV virus-vaccine between 1939 and 1946 in the Lake Albert plague focus, which has been so well investigated by van Riel, Mol, and especially our friend Devignat [120, 26]. It was a systematic detection of marine plague during rat-killing operations that had called for the vaccination, in order to prevent the appearance of human plague. In a table which sums up the results of this undertaking, we can see that out of 132 cases of plague authenticated between 1940 and 1946, 16 were recorded in vaccinated persons between 3 and 6 months after the vaccination, and 116 in non-vaccinated persons, the latter having become infected when there was no indication of the presence of murine plague which called for preliminary immunization. Devignat concluded that in "a native environment, vaccination by means of the virus-vaccine of Girard and Robic represents the most effective measure against plague." Fain et al. have continued to prepare the vaccine in the antiplague laboratory of Blukwa and to use it in the sectors where plague cases had been reported [33]. However, after the events which marked the accession of this territory to independence, we do not know whether there still is a plague control service in existence in the Congo. Nevertheless, during 1962, a South American physician accredited by the WHO began the preparation of the EV vaccine at the Pasteur Institute, before proceeding to Katanga.

In the USSR it did not appear, according to Korobkhova's work [87], that the epidemiological circumstances in any way called for the execution of mass vaccinations. In this respect the Russian authors were limited to the experimental studies. Nevertheless, an article by Osolinker [106] mentions that 508,008 vaccinations had been carried out with the EV vaccine in the Guriev region between 1943 and 1953.

Note. In a curious propaganda booklet written in French

and designed for a mass readership [22] we were surprised to read about the many precautions which had been taken during the performance of the first inoculations of the virus-vaccine in man. Five pages are devoted to this subject (pp. 239-244). Let us quote the beginning of this section: "In April 1939 Moscow finally gave permission to carry out a trial. It was decided to inoculate the 'EV' culture to three scientific co-workers: Korobkhova, Berlin and Tumanski, because during the last few years they had studied the properties of this strain more than anyone else. Doctor Yachuk injected 250 million EV microbes to each of the three doctors. The experimenters were isolated from the rest of the people; they were accommodated in the small building of the isolator (sic) Nobody entered this room except by wearing a mask and special clothes. If 'EV' should recover its old properties, it must not happen that the plague leaves the isolator. Hundreds of persons were concerned with the experimenters' fate, but those in the box were calm....." And we then come to the conclusion: "When the quarantine had ended, the co-workers of the Institute triumphantly received the doctors who have tested the action of the 'EV' culture on themselves. Everyone believed that it was his duty to shake their hand, to tell them something nice, to give evidence of their concern."

About this romanticised story, the writing of which is certainly foreign to our Russian colleagues, the least that can be said is that the inoculation of a live plague bacillus into man was considered to be a serious matter. Moreover, after Yersin's remarks, Robic and myself did not think it otherwise. However, in 1939 more than two million inoculations had been carried out in Madagascar, which the author of the above booklet seems to be unaware of. By contrast with this pusillanimous undertaking, other trials carried out without fanfare by Pokrovskaya and Kaganova were quite audacious: we certainly would not have undertaken them in Madagascar. These trials consisted in the administration by inhalation -- first to themselves and then to 20 volunteers -- of the EV virus-vaccine, as well as another live strain, AMP. We have mentioned above that the Russian authors were anxious to demonstrate the activity of the virus-vaccine in the protection of guinea pigs against pneumonic plague and that this protection was more durable if the vaccine was given directly in the form of an aerosol. It is after a very thorough histological study, to which we shall return, relating to the stimulation of the immunitary process of the lung, that the two researchers came to the conclusion that they could apply the method to man. According to their communication, these tests were not marked by any accident; there was no general reaction, no angina, no conjunctivitis. They consider the method inoffensive, and hope that it will make it possible to achieve victory in the fight against pneumonic plague [113].

In Tunisia, an epidemic of bubonic plague (37 cases) linked with murine plague raged in Ferryville between August 1944 and March 1945. Under the effective direction of Surgeon-General Le Chuiton, Director of the Public Health Service of the Maritime District, a vaccination was carried out first with killed vaccine, and then with live EV virus-vaccine prepared at the Pasteur Institute of Tunis. The live vaccine was administered to 59,301 persons. According to Magrou [96] who gave a detailed description of this operation, it is difficult to determine the role played by the vaccine in the evolution of this outbreak of plague which was concurrently attacked by deratization and intensive disinfestation by DDT; on the other hand, this was not an experiment in which valid non-vaccinated controls were also used. Our colleague nevertheless stressed that the cases of plague occurring in the vaccinated group were less severe than those which occurred in the non-vaccinated group.

In Senegal, where an offensive return of the plague took place in October 1944, about 100,000 inoculations with the EV virus-vaccine were carried out; however, even here it is impossible to say whether these vaccinations had any effect on the progress of this epidemic which, it seems, was already in its waning stage when the vaccinations were performed, the delay being due to a combination of circumstances which had delayed our departure to Dakar on this mission.

In Vietnam, the EV virus-vaccine had been used since 1946 when one or several cases of plague were identified. The reports of the Pasteur Institute of Saigon make mention of this vaccine which is being prepared in Saigon and Dalat where the EV strain is subjected to a regular control of its properties. However, only isolated cases of plague occur in Vietnam, and the problem of preventive vaccination is far from being as important there as it has been in Madagascar; notably, pneumonic plague is unknown in Vietnam.

Be that as it may, taking into account the facts observed in Madagascar, in the former Belgian Congo and in the Union of South Africa, it can hardly be denied that the EV virus-vaccine has contributed to the reduction of the risks of human infection wherever it was employed. It may have been of debatable utility and may have constituted only a precautionary measure during the last few years when the global epidemiological situation has evolved toward the spontaneous decline of plague, this decline being facilitated by the use of a systematic disinfestation; but a fundamental fact emerges from this immunization with the live EV vaccine, and let us add, with that of Otten in Java, and that is that not a single accident or even serious incident has been encountered in the course of the millions of inoculations performed during the last 30 years. Even if nothing more could be expected from the vaccine, this fact deserves being particularly stressed.

Discussion

Before entering upon the second part of this work, we must make mention of and reply to the criticisms which have been expressed with regard to the use of live antiplague vaccines in general, from the very beginning of their application, by Sokhey and Maurice of the Haffkine Institute of Bombay. These authors' experiments carried out under the conditions in which they worked led them to the conclusion that their vaccine killed by heat had a protective power that was 150 times greater than the live vaccine obtained artificially with the same strain [137]. To these allegations which related especially to the work of Otten who, as we have mentioned, had carried out the first tests on human subjects with an irreproachable discipline, according to the so-called alternant method, the Director of the Pasteur Institute of Bandung replied with a series of arguments which considerably reduced the importance which his two colleagues at the Haffkine Institute had attributed to their experiments [108]. Sokhey and Maurice had claimed that although the killed vaccines did not always fulfill their expectations, this was due to the fact that their sterilization was carried out at a too high temperature. For them, the suspensions should only be subjected to a temperature of 55°C for 15 minutes; in this way the vaccine protected both the mouse and the guinea pig. On the other hand, having cultivated the virulent strain from which they prepared their vaccine for six weeks at 37.5°C, they noted that its virulence was attenuated, then it disappeared, and it was this avirulent sample that they compared, as a virus-vaccine, with their killed vaccine. The comparison proved that the minimum dose protecting the mouse was 0.002 cm^3 of killed vaccine, and 0.3 cm^3 of live vaccine.

The uninitiated reader is obviously perplexed when confronted with this result, as presented by Sokhey and Maurice. However, we cannot accept the interpretation and the conclusion drawn by them any more than Otten did; our inability to accept the interpretation and conclusion is based on two reasons which, in our opinion, are very important ones:

1) Heating a suspension of virulent plague bacilli at 55° for 15 minutes (approximately 2×10^9 bacilli per cm^3) does not kill all the germs unless the suspension is subjected to a continuous agitation during the operation. While this vaccine protects the mouse against a test infection of mild severity, as do the majority of killed vaccines, it is completely inactive in the guinea pig. If the material was not agitated, a few live microorganisms may survive which a wide inoculation would make demonstrable, as has been shown by Robic. With this vaccine, which is apparently inoffensive for the guinea pig,

this animal will enjoy a certain degree of protection which may be attributed to the residual live germs [55]. However, for its human use, the Haffkine lymph was always treated with phenol (0.5%) in order to preserve it in such a way that the human subject always receives a perfectly killed vaccine. At the laboratory, a suspension heated to 55°C for 15 minutes and treated with phenol as above does not impart a trace of immunity to the guinea pig. Moreover, Otten stressed that the reading of the annual reports of the Bombay Institute reveals that the original vaccine prepared by Haffkine in 1897 was sterilized for one hour at 70°C; later, the sterilization was carried out at 65°, 60°, between 56 and 60°, and still these vaccines were considered by their authors as being highly effective on the basis of statistics whose interpretation has been so pertinently challenged by Otten after his experiment carried out in Java [107]. However, we owe it to the truth to point out that about 15 years ago, under the impetus of Sokhey himself, the technique of manufacture of the vaccine was perfected at the Bombay Institute [138, 139]; it is sterilized by means of formol, and this vaccine, as we have personally noted, imparts a high degree of protection to the mouse, while it remains without effect in the guinea pig, as are all the killed vaccines.

2) By assuming that the avirulent strain created by an experimental artifice from the same virulent strain must represent the ideal type of a virus-vaccine, Sokhey and Maurice attributed a general validity to the interpretation of an isolated experimental fact which confirmed what we had already known, namely, that certain avirulent strains are devoid of protective power. Had not Yersin noted that the quality of a live antiplague vaccine was linked to the maintenance of a certain virulence level [151]? We shall be more explicit with regard to the interpretation given to the term "avirulent" which is generally -- and wrongly -- used instead of the term "attenuated" or "weakened" virulence. We, ourselves, have pointed out that the "Pecha" strain, isolated in Vienna at the end of the last century, which for a long time was devoid of any virulence and which, as a result, was handled without risk in the laboratories by generations of students, did not confer a trace of immunity on rodents. In our first publications we claimed that the EV strain was the only one in a batch of cultures investigated whose stable characteristics justified the hope of using it for the preparation of an effective virus-vaccine. In 1937 Reitano [118] carried out a series of experiments with an avirulent strain (strain P) on the basis of the studies undertaken at Madagascar and Java, and concluded from the results, as Otten had concluded in Java, that among the avirulent cultures one encounters some that are endowed with a high immunizing power while others are completely devoid of

this power; he added that the reason for this contrast ought not to be sought "in the form or aspect of the colonies, but in certain other factors toward which the future investigations should be directed."

In truth, the mode of attenuation specified by Sokhey and Maurice had to lead to a degradation and then to the disappearance of the virulence of their strain as a result of a selection of mutants which are avirulent from the very beginning, mutants which exist in the majority, if not in all, strains of *Pasteurella pestis*. This selection is favored by culturing at 37°, and its effect is to cause the loss of certain antigens as was recognized by Fukui et al. [37]. This notion was unknown 25 years ago. The virus-vaccines EV and Tjiwidej were not obtained by culture at 37°, but quite on the contrary, and as for ourselves we have always advised the cultivation of strain EV at a temperature not exceeding 28°. Today it can be readily seen that a virulent strain which is in possession of its entire antigen complex preserves, after sterilization by the usual procedures (heat, formol), a part of its protective antigens, while a long series of cultures at 37° will lead to the selection of mutants entirely or partly deprived of these antigens. This is the meaning -- which could not be predicted by Sokhey and Maurice -- of their experiments whose results should not surprise us. However, in the theoretical objections of the Indian authors to the use of a live vaccine against plague there is an element which we must denote as a form of sentimentality. Loyal to Haffkine's lymph, the first antiplague vaccine, the sole vaccine which had up to then been prepared in a liquid medium, it was not without regret that the Bombay Institute deliberately accepted the abandonment of a technique which had been used for 40 years. Moreover, it may be objected that with a virus-vaccine it is difficult to build up stocks for an emergency as is commonly done with a killed vaccine. A rigorous and permanent control of the strain is imperative in order to prevent both a return to virulence and the accidents which would result from this, and above all, a degradation to the point of complete loss of immunizing power, examples of which we have encountered. We have never been unaware of these requirements and the constraints which they impose on us. We are inclined to believe that it is this group of arguments which was responsible for the bitterness which permeates the last lines of Otten's paper [108] written in reply to the criticism of Sokhey and Maurice which had been voiced during a meeting of interested workers, where our eminent colleague did not succeed in having his point of view accepted without reluctance: "That the Conference on Rural Hygiene, recently held in Bandung under the auspices of the Health Organization of the League of Nations, was unable to endorse this conviction, cannot by an insider be regarded as

surprising. In matters of medical hygiene, the Leage of Nations behaves as it does in politics: it strives only for compromise."

However, in the midst of the enthusiasm aroused by the first mass vaccinations in India, serious reservations were formulated by Simond and Yersin in their remarkable report on plague in the Far East, presented to the International Medical Congress in 1900 [135]. In the serenity of his retirement, our illustrious Old Man of the Colonial Medical Corps wrote us a letter in 1936 about this report; we mention the existence of this letter for the first time, and a photocpy of its essential passage is appended herewith.

Valence le 3 Mars 1936
15 rue de l'Espérance

Mon cher camarade

J'ai eu d'autant plus de plaisir à recevoir la série de vos importants travaux sur la peste et sur la vaccination par votre virus atténué, que l'obtention d'un vaccin efficace m'a paru la question capitale à résoudre en ce qui concerne la prophylaxie, depuis que j'ai expérimenté les difficultés de la défense par les autres mesures. Vous affirmez qu'il est impossible de conférer une immunité solide et durable au cobaye avec les vaccins tués. Je puis vous dire qu'en ce qui concerne le premier vaccin tué (autour duquel il a été fait grand bruit) celui de Haffkine, il en est chez l'homme exactement comme chez le cobaye. Témoin, pendant 2 années de la vaccination humaine par ce procédé dans une vingtaine de foyers indiens, sur des circonscriptions de mille habitants, j'en ai constaté l'échec par des observations si nombreuses et si précises que j'ai dû, dans mes publications, faire les plus expresses réserves sur les avantages de cette ~~[illegible]~~ vaccination. De ces observations j'ai tiré une grande leçon sur la facilité avec laquelle les auteurs d'un procédé non seulement se suggestionnent souvent eux-mêmes mais arrivent, par des statistiques non pas travaillées, à convaincre le public et même les techniciens de résultats inexistants. Et cela n'a pas lieu que pour les vaccins.

[Translation of letter on page 38]

Valence, 3 March 1936
15 rue de l'Esperance

My dear friend:

I was all the more pleased to receive the series of your important papers on plague and on vaccination with your attenuated virus since I always considered the obtainment of an effective vaccine to be the crucial problem to be solved in the area of prophylaxis, ever since I have experienced the difficulties of providing protection by other measures. You state that it is impossible to confer a solid and durable immunity on the guinea pig with killed vaccines. I can tell you that as far as the first killed vaccine is concerned (about which there was so much excitement), that of Haffkine, the situation is the same in man as it is in the guinea pig. The proof of this is that, during two years of vaccination of human subjects by this procedure in about 12 Indian foci, in several times 50,000 inhabitants, I have noticed the failure of this method by so many and so precise observations that I was able, in my publications, to express my deepest reservations on the advantages of this vaccination. From these observations I learned how easily the authors of a procedure not only frequently hypnotize themselves but succeed, by means of somewhat labored statistics, in convincing the public and even the technicians of the existence of nonexistent result. And this happens not only in the case of vaccines.

Part II

IMMUNOLOGICAL PROCESSES AND THE EV VIRUS-VACCINE

As we have stated in our introduction, it is only during the last 20 years that the plague bacillus has been the object of investigations of an immunological type, as a function of its antigenic structure. The Second World War gave rise to a series of studies in this area, especially in the US, whose troops were called upon to pass through and remain stationed in Asian and African territories which formed part of vast zones where plague was endemic. While understanding our action in Madagascar as being the result of particular conditions which had led to the adoption of the use of the EV virus-vaccine with results whose importance they never challenged, we noted in our conversations with our English-speaking colleagues the existence of a reservation, in principle, toward all live antiplague vaccines. Thus, one of their principal objectives was to perfect immunization by making an effort to extract from the cell of *P. pestis* the protective antigens which would be capable of advantageously replacing the classic vaccines, all of which were composed of killed germs. On this basis they were necessarily led to undertake an experimental comparison of the immunitary mechanisms of the various types of vaccines, including the virus-vaccines, and among the latter notably those which had been applied to human subjects on a vast scale: the EV strain in Madagascar, and the Tijiwidej strain in Java. Two teams of researchers were particularly deeply involved in this task: first in the US that of the George William Hooper Foundation in San Francisco under the direction of K. F. Meyer, and later, in England, that of the Porton Research Laboratory for Medical Microbiology under the direction of T. W. Burrows. Their work will be exhaustively treated in the present article, but first, it is indispensable that we give a precise definition of the terms to which our English-speaking colleagues attribute meanings that are not identical to those commonly adopted in France. In this way the reader will be better able to interpret the critical analysis to which we shall subject these papers which, even though restricted to the experimental field, nevertheless span an exceptionally broad range of topics and mark an epoch in the history of immunology.

Virulence, Toxicity, Pathogenic Power in *Pasteurella pestis*

Pacing M. Nicolle, we consider that the virulence of a microbe resides in its ability to multiply in the organism more or less abundantly and to create lesions in the latter. We shall add that these lesions may be transitory and have no apparent effect on the behavior of man or animal, in which they may be called "reactions" rather than lesions. While these manifestations are no doubt attributable to poisons elaborated by the microbial agent, experimentation teaches us that the toxic power is not necessarily parallel to the degree of virulence of the plague bacillus. It is these two properties -- virulence and toxicity -- whose combination defines the pathogenic power of the agent. This notion is not expressed distinctly in the publications of the American scientists for whom "virulence and pathogenicity" are most frequently synonymous. We have underlined these differences in a previous paper published in English which we had been requested to write in order to give an up-to-date account of the progress made in the study of plague and of its etiological agent during the last two decades [56].

This distinction between virulence and toxicity appears in the mode of reaction of laboratory rodents to experimental plague. The guinea pig, for example, is insensitive or almost insensitive to the toxin, and succumbs only after an intensive proliferation of the virus which multiplies in the blood and the organs, notably the liver and spleen. By contrast, the white rat is killed more rapidly with the same strain if this strain is very toxic, and there will be very few germs in the tissues. However, if the culture is not too toxic and if the inoculation comprises only a small dose of cells, the rat will be able to resist while in the guinea pig, an ideal reagent of virulence, the disease will last only for a few days and then have a fatal outcome, according to its usual manner. It is these observations made with material taken from human cadavers that have led us, with Robic, to use only the guinea pig for the control operations of the post-mortem detection of plague in Madagascar. The white mouse, in turn, is the animal of choice for the studies of the toxin to which it is highly sensitive, while at the same time it offers to the plague bacillus one of the most propitious media for bacillar development if it does not succumb too rapidly to the intoxication.

The absence of any correlation between the two factors which control the pathogenic power of *P. pestis* is clearly demonstrated in the case of certain so-called avirulent strains from which one can extract a toxin whose activity is in no way

inferior to that of the most virulent strains.

In the first part of this work, where we mentioned the essential characteristics attributed to the EV strain, we stressed the fact that it remained toxic, and that we believed that it probably owed its protective power to the persistence of this property. Our opinion was reinforced by our subsequent investigations on certain protective avirulent strains while one strain devoid of any immunizing power was atoxic [57]. This point of view has been refuted -- as we shall see later on -- by Meyer et al., who believe that the toxic fraction may be eliminated in the killed vaccines of the protective antigenic complex to which the toxic fraction is supposed to be foreign [99].

Furthermore, let us note in connection with the plague toxin that the toxin which is extracted from the protective avirulent strains has been found to be identical to that of the virulent cultures. Thus, Goodner, studying the optimal conditions of the production of the toxin, has worked with the EV strain [75]. Spivak and Karler have studied the virulent Indian strain 195/P and the EV strain; the toxins of both these strains behaved similarly [140]. Ajl et al. have obtained a highly purified toxin from the avirulent Tjiwidej strain [1]. The respective independence of the virulence and toxicity can hardly be doubted in the case of *P. pestis* whose pathogenic power is completely subordinated to the "virulence" factor; however, the interpretation which we give to this factor is less rigid than that of our American colleagues, because it is based on experimental observations which they themselves have confirmed.

The publications of these authors mention only two types of strains of plague bacilli: those which are pathogenic under conditions which, in truth, have been arbitrarily established, and those which are not pathogenic; they regard the first as virulent and the second as avirulent. Nowhere is there any question of strains of attenuated or weakened virulence, a qualification which is met precisely by the EV and Tjiwidej virus-vaccines and probably also by those which have exhibited a real activity and whose study has not left the confines of the laboratory. In the above-mentioned paper [56] we have stressed this gap in the vocabulary, by calling attention to a paper of Englesberg et al., for whom a strain is virulent if it causes the death, by septicemia, of a mouse in which a dose not exceeding 300 germs has been subcutaneously inoculated. A strain is said to be avirulent if 10,000 or more germs are tolerated by this animal, a criterion which is satisfied by the EV and Tjiwidej strains.

For Burrows, a strain is virulent if 20 cells inoculated subcutaneously kill the mouse, and by a peritoneal route, the guinea pig; he considers those strains avirulent which are pathogenic only at a higher dose; however, in a recent publication in which the author treats this very complex problem of

virulence in its entirety [12], he takes care to stress that the term "avirulent" comprises the strains of reduced virulence whose L_D varies between 10^2 and 10^7 (thus, over a large range) as well as those for which the L_D exceeds 10^8 for the mouse and 10^{10} for the guinea pig. We are inclined to assume, because there is nothing explicit on this point, that only the latter strains would meet the criteria for being designated as true avirulent strains. The notion of attenuated virulence was imposed on Burrows when, following certain patient researches, he specified the characteristics which permit the determination of the virulence in *P. pestis* in vivo. We shall discuss this in a chapter devoted especially to the work of the Porton school.

Note: The term "attenuated virulence" is now used for all microbial strains devoid of pathogenic power when administered under given conditions, when under the identical conditions the cultures from which they were prepared were originally pathogenic. The distinction established by M. Nicolle between attenuated virulence and weakened virulence, according to whether or not this state is durably fixed, is no longer taught to the new generations of microbiologists, most of whom are unfamiliar with this distinction. Hence, when we speak of attenuated virulence in the following part of this work, we shall have the general meaning of this form in mind in order to conform to current usage, even though it is doubtful if there are strains that are truly attenuated, as defined by M. Nicolle [105].

Pasteur himself, as is appropriately recalled by G.Ramon in *Pages d'Histoire de l'Immunologie* (Pages from the History of Immunology), has had to "recognize under the pressure of facts that the virus-vaccines whose virulence was not abolished but only reduced, are not fixed in their properties, and that the latter may become modified under certain conditions." *This Journal*, Jan.-Feb. 1962, 51, p. 16.

Demonstration of the Partial Virulence of Strain EV

We have never claimed that strain EV was avirulent. In our initial description of its essential characteristics in 1933 (see the first part of this article), we gave the reasons which had led us to regard it as a strain of weakened virulence, and a toxic one as well. In truth, the experiments carried out on guinea pigs which had been inoculated with many billions of live germs intraperitoneally would have led us to attribute the death of those animals which had succumbed to this massive inoculation rather to the combined action of the two elements of pathogenic power: virulence and toxicity. The guinea pig is very little sensitive to the toxin extracted from cultures in vitro, but in vivo the plague bacillus produces in this animal a toxin that is much more active, as has been shown in 1957 by

Keppie et al. [83]. Under our experimental conditions, the toxin which weakens the resistance of the animal would permit an intense and rapid proliferation of the etiological agent.

The proof of the persistence of a certain virulence of the strain was provided when we reduced to around 1 billion germs the dose administered to a series of guinea pigs, two of whom were sacrificed daily for a period of 30 days. In effect, we have noted in several animals inoculated intraperitoneally and sometimes subcutaneously, the presence after a few days of nodular reactions in the spleen, more rarely in the liver. Our late lamented colleague Jean Bablet subjected these lesions to a study [6], whose essential findings are as follows:

From the macroscopic point of view we note, from the seventh day on, a hypertrophy of the spleen which may attain twice its normal volume. The surface of the organ acquires a rough and rugose appearance. On the tenth day or so there consistently appear some granulations whose maximum size is that of a pin, and which stand out against the violet background of the organ by their grey color. On the 15th day they begin to fade out and disappear between the 20th and 25th day, leaving behind a small, hardly visible scar. On the 30th day everything is normal again, and the spleen has returned to its normal volume.

Histological examination reveals after the fifth day the presence of small nodular formations in the red pulp, made up of a dense mass of reticular cells centered by a group of polynuclears containing a few coccus bacilli. During the following days the lymphoid formations of the white pulp fade out somewhat as a result of the proliferative thrust of the reticular and endothelial elements. Around the tenth day the reaction phenomena attain their peak, the congestion is intense and the reticular nodules contain more or less disintegrated polynuclears, bacillar debris and live bacilli in their center (as is demonstrated by the inoculation into mice). These formations are rather similar to the reactional nodules produced in the organs by the experimental i.v. inoculation of large doses of BCG bacilli. Regression begins after the tenth day. On the 30th day the nodules have disappeared; only a reticular hyperplasia of the cords remains; the white pulp has resumed its normal appearance.

If, under the same conditions, we inoculate the guinea pig with EV bacilli killed by heating to 60° -- these bacilli nevertheless remain toxic -- no reaction takes place in the spleen, nor is there any reaction with virulent bacilli likewise killed by heating. Hence, the nodular reaction is linked to the introduction of live bacilli into the organism, and it causes the segregation in the splenic pulp of these bacilli whose toxigenic power does not play a part in the mechanism of this reaction. Attenuated strains of plague bacilli of weak

immunizing power are incapable of causing the appearance of splenic or hepatic granulations. Hence, the latter would have the value of a control reaction to check the immunizing qualities of a plague bacillus.

In another series of experiments in which the guinea pigs were inoculated -- this time subcutaneously -- with the same dose of germs [109], we have attempted to isolate the EV bacillus from the principal tissues or organs with our collaborator Radaody-Ralaros, who was guided in this trial by the results of his previous work on the fate of BCG, inoculated into the guinea pig. The animals were sacrificed after 3, 6, 9, 15 and 24 hours, then after 2, 3, 4, 7, 9, 11, 13, 16, 21 and 29 days [58]. The following findings were made:

Only a small quantity of EV bacillus circulates in the blood, where it was detected only during the 40th hour, never afterwards. It could only be isolated from the organs after two days. The spleen, which was the first organ to be invaded, retains a maximum amount of this bacillus on the fourth day, and no longer contains any after the 11th day. The liver is invaded only somewhat later, but it is in the hepatic tissues that the bacillus lingers for the longest time (15 days). It was encountered once in the axillary ganglion but never in the bone marrow. Although smears have demonstrated its passage to and persistence on the level of the lung, the extreme proliferation of the associated banal germs did not permit us to isolate it from this organ.

In the table appended to said work, we shall note that a count of the colonies isolated in the spleen dilutions would show, for the whole organ, at least 60,000 germs between the third and fourth days, and 2500 in 1 g of hepatic tissue on the 11th day.

The preceding experiment was repeated with the Pecha strain, which is quite avirulent and does not possess the least protective power. At no time was its bacillus encountered either in the blood or on the level of the principal organs.

It may be objected that these experiments attest only that the EV bacillus is not immediately destroyed in the organism, contrary to the avirulent plague bacilli devoid of immunizing power, and that its presence in the tissues for a variable period of time does not necessarily imply that it is multiplied there, while assuming that this presence is responsible for the high protective power conferred by the virus-vaccine, due to the reactions which it gives rise to.

This is a point of view which Otten readily subscribed to. He recalled that Strong had stressed during his attempts at vaccination with a live bacillus, which were followed in 1908 by 200 human vaccinations (without any incident) [142], that in the monkey, the virus proliferated rapidly at the inoculation site after the sixth hour, but that it was no longer detectable in culture after 24 hours. He did not look for it in the viscera. This multiplication in situ which he estimated at a

immunizing power are incapable of causing the appearance of splenic or hepatic granulations. Hence, the latter would have the value of a control reaction to check the immunizing qualities of a plague bacillus.

In another series of experiments in which the guinea pigs were inoculated -- this time subcutaneously -- with the same dose of germs [109], we have attempted to isolate the EV bacillus from the principal tissues or organs with our collaborator Radaody-Ralarosy, who was guided in this trial by the results of his previous work on the fate of BCG, inoculated into the guinea pig. The animals were sacrificed after 3, 6, 9, 15 and 24 hours, then after 2, 3, 4, 7, 9, 11, 13, 16, 21 and 29 days [58]. The following findings were made:

Only a small quantity of EV bacillus circulates in the blood, where it was detected only during the 40th hour, never afterwards. It could only be isolated from the organs after two days. The spleen, which was the first organ to be invaded, retains a maximum amount of this bacillus on the fourth day, and no longer contains any after the 11th day. The liver is invaded only somewhat later, but it is in the hepatic tissues that the bacillus lingers for the longest time (13 days). It was encountered once in the axillary ganglion but never in the bone marrow. Although smears have demonstrated its passage to and persistence on the level of the lung, the extreme proliferation of the associated banal germs did not permit us to isolate it from this organ.

In the table appended to said work, we shall note that a count of the colonies isolated in the spleen dilutions would show, for the whole organ, at least 60,000 germs between the third and fourth days, and 2500 in 1 g of hepatic tissue on the 11th day.

The preceding experiment was repeated with the Pecha strain, which is quite avirulent and does not possess the least protective power. At no time was its bacillus encountered either in the blood or on the level of the principal organs.

It may be objected that these experiments attest only that the EV bacillus is not immediately destroyed in the organism, contrary to the avirulent plague bacilli devoid of immunizing power, and that its presence in the tissues for a variable period of time does not necessarily imply that it is multiplied there, while assuming that this presence is responsible for the high protective power conferred by the virus-vaccine, due to the reactions which it gives rise to.

This is a point of view which Otten readily subscribed to. He recalled that Strong had stressed during his attempts at vaccination with a live bacillus, which were followed in 1908 by 200 human vaccinations (without any incident) [142], that in the monkey, the virus proliferated rapidly at the inoculation site after the sixth hour, but that it was no longer detectable in culture after 24 hours. He did not look for it in the viscera. This multiplication in situ which he estimated at a

its effectiveness during the controls, to which it was regularly subjected during its use in human immunization.

In fact, the extensive studies carried out on the antigenic structure of the plague bacillus gradually revealed the existence of certain basic differences between several "avirulent" protective strains and, from this point of view, of a very particular behavior of the EV strain.

Antigenic Structure of *Pasteurella pestis*.

Relationship Between Fraction 1 and Immunizing Power

Prior to the work of the American scientists, Schutze [132] showed that the plague bacillus possessed two antigens, one corresponding to what he called "envelope" (today it is considered a capsule) and the other the somatic antigen. The former, unstable to heat, attained its maximum development in 37°C cultures, whereas the latter, stable to heat, formed equally well at 20° and at 37°. Furthermore, we have reported [59] that Boivin's trichloroacetic acid technique did not make it possible to demonstrate the existence of a complete antigen (fat-carbohydrate-protein) in *P. pestis*; later, in a paper coauthored with Sandor, we pointed out that the plague toxin, which has an exclusively proteinic composition, behaved rather like an exotoxin despite the fact that it is liberated by microbial lysis, and that it occupied an intermediate place between the true exotoxins and the endotoxins [60, 129].

We owe the first statements on the antigenic structure of *P. pestis* to Baker et al. [8] (1947). Working with massive suspensions of germs cultivated on agar, then dried and treated by a suitable technique, these authors isolated three characteristic fractions from it: Fraction 1, which is nontoxic, itself breaks down into two subfractions: 1A, a protein combined with a polysaccharide, and 1B, protein alone; however, since these two substances have common properties, we shall henceforth refer to them as F 1. When injected in the rabbit, F 1 causes the formation of agglutinins against the total microbe; the obtained serum protects the mouse against the infection but does not have any neutralizing effect vis-a-vis the toxin. This antigen immunizes the mouse and the rat but not the guinea pig. It is produced especially in cultures of 37°C by the virulent and certain protective avirulent strains. On the whole, it corresponds to the antigen of Schutze's "envelope." A second fraction, F 11, is toxic for rats and mice; it is antigenic and the rabbit antiserum neutralizes the toxin but only agglutinates *P. pestis* to a slight extent or not at all. Fractions 1 and 11 are soluble in water. A third fraction, included in the insoluble residue, is made up of proteins which contain an antigen

which is protective for the guinea pig. Subsequently, Seal in Calcutta [133] and Amies in Johannesburg [2] have confirmed, with the aid of original techniques which differed from that of Baker, the presence of similar antigens in fraction 1 in the capsular substance which represents 2 percent of the dry weight of the microorganism which, according to Amies, is composed of proteins combined with a small amount of hydrocarbon and whose solution possesses strongly immunizing and precipitating characteristics. A few micrograms of purified antigen suffice to confer a solid protection on a sensitive African rodent, *Mastomys natalensis*. However, the authors have failed in their attempt to discover an antigen of type Vi which would have permitted to distinguish the virulent from the avirulent strains by means of serological tests.

It is around this fraction 1 that the work of Meyer's school was carried out. For Meyer the value of an antiplague vaccine -- whether live or killed -- is a function of its content of this antigen, and of this factor alone. Highly purified by a series of operations which we shall not go into here [9], this fraction immunizes rats, mice and monkeys but not the guinea pig; however, these authors were not too concerned with this fact since they considered that the behavior of man is closer to that of the mouse than of the guinea pig. The argument advanced in order to shore up this concept is that man, like the mouse and unlike the guinea pig, is sensitive to the plague toxin. We consider this argument rather specious in the form in which it is presented. While intoxication plays a dominant role in the symptomatology of human plague and while the guinea pig hardly reacts to an injection of toxin, it should, nevertheless, be stressed that we are dealing with a toxin which is obtained by culture in vitro; we shall see later on that the situation is not the same with a toxin elaborated in vivo, particularly in the case of the guinea pig. Be that as it may, fraction 1 is allegedly sufficient by itself to protect man against plague. While fraction 11 which is toxic does have antigenic properties, it is of no use, which makes it possible to inject a purified, atoxic antigen representing, in a small volume the active element contained in tens of billions of total germs which could not be administered to man without danger, owing to their toxicity.

It cannot be doubted by anyone examining the experimental records and the numerical results of our colleagues from San Francisco that there exists a relationship between the F 1 content and the immunizing power of a virus-vaccine [99]. Out of approximately 15 so-called avirulent strains we note that strain 1122, which has the highest content of F 1 (15-18 percent of the capsular antigen) protects 50 percent of the mice against a standard infection with only 300 cells. With strain 343 (from the Congo) whose F 1 content is 2.3 percent, 90,000

cells are necessary; with the Tjiwidej strain (1.5 percent F 1) the figure rises to 400,000; with strain 14, whose F 1 content is less than 0.02 percent, the dose attains 30,000,000 cells. This correlation is even further strengthened in the case of two samples of strain EV, one of which (EV 76 old) was obtained by Meyer via an intermediary (?) and the second (EV 76 Girard) used by ourselves after control of its properties. The former contains only 1.9 percent F 1, and the dose which protects 50 percent of the mice is 1,400,000 germs. The F 1 content of the second is 5 percent, and the same result is obtained with 200-500 cells. In the case of the guinea pig these findings are different, and the strain which protects 75 percent of the guinea pigs with 30,000 cells requires several million cells to immunize the mouse. The contrast which we find between these two samples of EV is just as significant for the guinea pig as for the mouse, since 3 billion cells of the former strain (EV 76 old) are needed to protect 80 percent of the guinea pigs, and 50,000 of the second (EV 76 Girard) to protect 100 percent of them. Strain 1122, which is so rich in F 1, immunizes the guinea pig only after inoculation of 300 million germs.

Hence, for Meyer the ideal procedure would be to vaccinate man solely with the purified antigen F 1; however, its preparation is a delicate and costly undertaking; hence on the practical level, 37°C culture suspensions of virulent or non-virulent strains may be used as "total" vaccines exhibiting an effectiveness which is comparable to that of the live vaccines if they are killed and detoxified with formol, provided that these strains are rich in F 1. However, when fraction F 1 was incorporated into adjuvants such as falba or bayol F, Spivak et al. [141] rather unexpectedly succeeded in immunizing the guinea pig, at least to a partial extent, without the participation of the insoluble residual fraction which until then had been regarded as the specific antigen which protects this rodent. Some anti-F 1 antibodies were detected in the serum of guinea pigs vaccinated in this manner. This serum had a certain protective action in the mouse, a property which is manifestly linked with the presence of this antibody, since the absorption of the serum by purified F 1 made it disappear. During these investigations, these authors were surprised to note that a dose of 5 mg of F 1 emulsified in the oily adjuvant failed to protect the guinea pig, whereas the protection was excellent (these are their own words) with 0.005 mg; this is a phenomenon of immunoparalysis, which was investigated in a series of papers by Walker [147] and compared with the phenomenon described by Felton and Ottinger in the case of the pneumococcal polysaccharide in mice. In view of these results, Spivak et al. no longer assume the existence of a special antigen which exerts a specific protective action in the guinea pig.

Discussion. Considered from this angle, the problem of immunization appeared in a new light and became simplified, by uniting the killed vaccines and the live vaccines in the same process. Without ignoring the major role played by fraction 1 of the surface antigen and the recommendation to prepare the cultures at 37° to obtain the maximum yield of this fraction, it may be objected that the vaccine of the Bombay Institute, which is no doubt effective in the mouse, originated from cultures of 28°, as did its predecessor, Haffkine's lymph. As for ourselves, we have always recommended that strain EV be cultured at 26-28°, both for its maintenance and for the preparation of the live vaccine. If the quality of this vaccine depended on its F 1 content, it is hardly explainable why a small dose of live germs confers solid protection on the mouse while a considerable dose of the same germs whose vitality has been destroyed by heat or formol has practically lost its effectiveness. The F 1 content of EV (5 percent) is considerably lower than that of strain 1122 (15-18 percent), and still the mice are protected with a similar number of cells. In the case of the live vaccines, Spivak et al. [141] recognize that the differences noted in the immunizing power of the various avirulent strains -- an observation which we have underlined from the very beginning of our studies, and which resulted in our retaining only the EV strain out of the many strains investigated -- cannot be explained on the basis of the antigenic composition alone: other factors must intervene, primarily the multiplication of the microorganisms and their persistence inside the tissues; and they add that as long as these individual factors have not been clearly elucidated, the avirulent strains to be recommended for the immunization of humans will have to give evidence of their protective power both in the mouse and the guinea pig. According to them, two strains met this requirement at the time of their publication (1958): the EV strain and a strain called B 1456-4. Let us note in passing, before going deeper into the question of the preservation of the properties of virus-vaccines that, 30 years after our first acquaintance with strain EV, the latter has completely preserved its original characteristics.

Meyer and Foster [100] have drawn their argument from the presence of antibodies in the serum of persons vaccinated with fraction 1 or the total vaccines combined with the oily adjuvants, in order to evaluate the degree and the duration of the immunity conferred in this manner. Like most authors, they, too, consider that the agglutination and complement-fixation reactions are too random to have any value, but that a mouse sero-protection test (mouse protection index, MPI) whose technique and mode of interpretation they describe, has a veritable meaning. In non-immunized volunteers who had received three injections of purified F 1, the serum contains notable quantities of protective antibodies. The concentration of these

antibodies is smaller with three injections of total vaccine or one inoculation of live vaccine (strains 1122 and Tjiwidej). From these investigations they conclude that 1) the amount of protective antibodies in the serum decreases rapidly, and in order to maintain it at an appreciable level it is necessary to carry out repeated injections of vaccine, which stimulate the production of a higher concentration of antibodies than was achieved with the first vaccination; 2) certain individuals are unable to produce antibodies, even if they are subjected to several repeated injections of antigen.

Favarel, in Madagascar [34], has sought to evaluate the protective value of the various sera of persons vaccinated with the EV virus-vaccine or who have recovered from an attack of plague, with or without treatment, using a sero-protection test inspired by the MPI. The test to which the mice were subjected was more severe than that adopted by Meyer. Our colleague "has demonstrated certain differences in the value of the immunity which follows the vaccination and that conferred by the disease, and for each of the modalities he noted certain variants depending on the intensity of the vaccinal reaction and on the repetition of vaccination in the former persons and on the form of the disease (bubonic or pulmonary) in the latter."

Spivak et al. postulate, in favor of the protection of the guinea pig attributed to fraction 1 + adjuvants, the presence of large amounts of agglutinating antibodies, sensitizing agents and especially hemagglutinins.

However, it has not been proved that an effective immunization necessarily accompanies this production of seral antibodies. We have detected only insignificant amounts of agglutinins in our guinea pigs which were solidly protected by our live vaccine, even after the severe virulence test to which they were subsequently exposed. As for the proteinic hemagglutination (HA) reaction, a technique developed by Neel and Baltazard has shown that this method did not permit evaluating the degree of immunity. To be sure, this is a specific and highly sensitive reaction, but in agreement with Neel [103] we have found it positive at levels of the same order of magnitude in guinea pigs vaccinated with a killed vaccine (without adjuvants) when the animals had not been given any protection as in those who were effectively protected by the EV virus-vaccine. For Neel, who carried out a study of the HA, the latter only shows, when it is positive, that the organism has been in contact with the surface antigen of the plague bacillus; it has an undoubted epidemiological interest but cannot substitute the sero-protection test of Meyer and Foster. It cannot be doubted that the addition of adjuvants to the killed vaccines or to fraction 1, with the local reaction which these adjuvants give rise to, increases the concentration of these antibodies while at the same time stimulates the immunitary processes, as has been

shown by Ramon about 40 years ago in the case of horses used for the production of diphtheria and tetanus antitoxins. This is clearly shown by Spivak's experiments in the guinea pig, which we have mentioned above. However, we have never cared to suspend our live vaccine in anything but physiological saline solution, and this is perhaps the reason why, despite the local reaction which the inoculation of this vaccine entails, seral antibodies are rare or nonexistent, even though the guinea pig is highly immunized. It is expedient to stress, in addition, that the American authors administer three injections of total vaccine or of fraction 1 at one-week intervals, and test their animals three weeks later. Hence, the immunity is acquired only belatedly, while in the case of the EV vaccine it is already appreciable on the fifth day and is total on the seventh day following the inoculation.

In a major article in which Meyer analyzed the pathogenesis of plague, the natural resistance to infection and the mechanism of defense in acquired immunity [101], the author, describing the work in which his school has so intensively participated, admits that the concentration of antibodies in the serum of a human subject who had recovered from the disease, or in that of animals immunized with specific antigens or with avirulent strains, is sometimes too low to be detected by the usual tests, including the MPI. Despite the fact that these antibodies may be characterized for several months in certain cases, Meyer adds that their presence is not necessarily an indication of the degree of immunity acquired. Cellular factors play a predominant role, and this applies singularly to the cells of the regional lymphatic ganglia and of the local inflammatory exudate in natural plague or after vaccination, on contact with the antigenic proteins. It is significant, he writes, that the immunity following the infection is stronger than that conferred by an avirulent strain which, in turn, is more *durable* (our italics) than that produced by the antigens alone. Although it remains to be proved that the infection in an immune animal stimulates the physiological activity of the histiocytes by producing antibodies more rapidly than in the sensitive animal, Meyer is inclined to believe that the level of the subsequent immunity is conditioned by an increased phagocytic capacity of the microphages and macrophages. Various observations seem to indicate that "the individual resistance to plague infection depends on the physiological activity of the mesodermal tissues and not on some mysterious lytic humoral antibody."

The Bases of the Virulence of *Pasteurella pestis*

Until the work of Burrows and Bacon which we shall analyze later on because of its importance for our topic, we were

in complete darkness as to the elements which were responsible for the virulence of *P. pestis*. For Englesberg et al. [32] the virulence must be a function of at least two factors: 1) the envelope (capsular) substance which protects the microorganism against phagocytosis and probably blocks the action of the antibodies on the cell due to the solubility of this substance, and 2) the production of toxin which, in the last analysis, is responsible for death in plague infection. However, certain avirulent strains are capable of producing both capsular and toxic substance, and the aforementioned authors believe that the virulence of a given strain depends only on the quantity of these substances. They verified their hypothesis by determining the antigen of the envelope (fraction 1) and titrating the latter's toxin in 16 virulent and 9 avirulent strains; on the basis of the inferences made from these operations they defined the virulent strains as those which kill the mouse after inoculation of 300 germs, and the avirulent strains as those which leave the mouse unaffected after the inoculation of 10,000 germs. We have stated above that this distinction was quite arbitrary. The virulent strains produce more envelope substance than the avirulent ones; nevertheless, the limit between the two types whose F 1 and toxin concentration might be similar was difficult to explain and these authors expressed the view that certain unknown factors must have intervened in this differentiation.

Burrows and Bacon, in turn, addressed themselves to this problem and opened up highly interesting new paths by extending our knowledge of the factors by which virulence is determined.

While they assume [13] that certain avirulent strains of *P. pestis* differ from the virulent strains by the loss of their capsular antigen, there are also other avirulent but protective strains which possess, in vitro, characteristics that are identical to those of the virulent strains. The antisera prepared with either of these strains have similar properties. Hence, it is only by their behavior in vivo that they can be differentiated.

Burrows and Bacon first turned to the study of phagocytosis, by carrying out a series of experiments with two strains of *P. pestis*, one virulent and the other avirulent and protective (Tjiwidej) whose fate they followed in the peritoneal exudate of the mouse. Their findings support those which Meyer -- in addition to other authors whom he names -- had made in the inflammatory exudate following subcutaneous inoculation, and of which the microphotographs presented in his paper [101] give an eloquent illustration. These results may be summed up as follows: During the first few minutes after inoculation, the phagocytic activity is identical in both cases, but soon the number of bacilli undergoing phagocytosis varies in inverse proportion to the virulence; by contrast, the peritoneal exudate

is rich in free virulent microbes, whereas the number of avirulent ones in it is small: 10^7 and 10^3, respectively. The authors believe that this resistance of the virulent microbes to phagocytosis has to do both with a modification of their constitution and to an inhibition of the polynuclear leukocytes (the only elements which they have taken into account, because they represent 95 percent of the leukocytes in their experiments). They expect that their subsequent experiments will solve these problems.

Factors Responsible for Virulence. Primacy of the "VW" Antigen

Burrows and Bacon transposed the in vivo experiments whose conclusions we summed up above to in vitro experiments. They confirmed that by means of "phagocytic" tests involving the polynuclear cells of the mouse, the virulent strains may be differentiated from the protective avirulent ones. Cultivated at 28° on nutritive agar, the two types are highly sensitive to phagocytosis, but if the organisms are incubated for three hours at 37° in a broth culture, the resistance increased notably only in the case of the virulent strains [14]. It further follows from these studies that the behavior of the strains of *P. pestis* with respect to this phagocytosis is independent of the presence or absence of capsules, which represents the first reservation regarding the hypothesis of Meyer et al., for whom the pathogenic power is linked with the antigenic structure of the capsular substance. They envisage the possible existence of an antigen specific to the virulent strains. Incidentally, they discovered that the virulent germs which undergo phagocytosis are no longer viable.

However, an essential and novel fact soon dominated the continuation of the work of Burrows and Bacon: the demonstration of the existence of two antigens, V and W, whose presence is characteristic of the virulent strains and is associated with their resistance to phagocytosis, so that these antigens are similar to the Vi antigen of the Salmonellae [15]. These antigens are produced by cultures of 37° both in the case of encapsulated and non-encapsulated strains. The antisera prepared in the mouse and the rabbit with various strains inoculated in the live state, regardless of their degree of virulence, increase their sensitivity to ingestion by the polynuclear leukocytes of these two species when these sera are placed in contact with germs resistant to phagocytosis. While the saturation of these antisera by resistant germs or germs sensitive to phagocytosis gives rather aberrant result which did not permit the authors to formulate any conclusions in this area, one fact is firmly established: these antigens are, to a great extent, independent of fraction 1 of Baker [9] because an

antiserum which sensitizes the germs to the action of the phagocytes preserves this property if it is saturated by this fraction 1. An antigenic analysis by means of Ouchterlony's precipitation-diffusion technique has made it possible to locate two lines corresponding to the two antigens, V and W, in question, of which the second may be absent but could never exist in the absence of the first. Hence, we combine them under the designation "antigen VW."

Stressing in a short review [16] that certain strains may be virulent even though devoid of envelope antigen an of fraction 1, provided that they contain the antigen VW, Burrows wondered whether the trust placed in fraction 1 for protecting man against plague infection had not been exaggerated.

This discovery ought to be of direct interest to us, considering that of all avirulent strains of varying degrees of protective power which we have studied in the laboratory, the EV strain was the sole exception, since it behaved like the fully virulent strains by its marked resistance to phagocytosis and by its possession of antigen VW.

By this fundamental characteristic which, for Burrows, provided a sharp distinction of strain EV from the group of protective avirulent strains, we were inclined to consider that the permanence of antigen VW in its makeup is responsible for its high immunizing power in the singular case of the guinea pig. Referring to our first studies during which we compared the properties of our strain with those of Otten (Tjiwidej) since they were the only ones employed for humans in the form of a virus-vaccine, we now understood why the Javanese strain, which gave more consistent results in the mouse and rat than the Malagasy strain, was much inferior to the latter in the case of the guinea pig. Even two weeks after the inoculation of the vaccine the animals had developed only a PI*, and never a TI following the severe virulence test defined at the beginning of the present study. After two or three months the result was reflected by an AI [61]. This strain, like ours, was toxic, and if at that time** we had known about fraction 1 of the capsular

*For the meaning of these abbreviations we refer the reader to the first part of this paper.

**We are referring to the period (1934-1937) when Otten published his studies and vaccinated the population of Java with his live vaccine. In effect, after the premature death of our colleague, the Tjiwidej strain has passed through many hands and, after what we have read, one cannot rely too much on certain statements which make one assume that certain variations have manifested themselves in the subcultures studied in the laboratories. After all, has not the Tjiwidej strain been sometimes classified as glycerol positive when it, like the EV strain, belongs to the oceanic, glycerol-negative type?

We shall express ourselves more explicitly later on regarding the sometimes considerable modifications which the strains of *P. pestis* undergo depending on the method of preservation adopted in order to preserve their initial properties to the greatest possible extent.

substance, it is possible that we would have found it present in this strain in a high concentration, in view of the protective effect exerted by this strain in the mouse. Likewise, it is to the presence of antigen VW that we can attribute the more pronounced transitory multiplication and the longer persistence in the tissues of the germs originating from the EV strain, compared with those of the Tjiwidej strain, as we have already reported in our analysis of a paper by Walker et al. [146].

The agreement between our experimental results regarding the properties of our respective strains led Otten, in 1936 [107], to predict the existence of at least two immunizing antigens in these virus-vaccines: one which is active especially in the guinea pig, and the other in the rat. A practical conclusion was drawn from this: in view of our ignorance regarding man's behavior vis-a-vis the plague infection, an ideal vaccine would be one that would immunize the two rodents equally well, and Otten added: "It is possible that a combination of two or more strains will be indicated in the future in order to provide a better protection for man than he currently enjoys." This view, which we have shared [62], was recalled here in homage to the memory of the Dutch scientist whose point of view was amply justified by work carried out during the last 15 years.

Other Factors Responsible for the Virulence of *P. pestis*

After showing that the virulent strains cultivated at 37°C possess, in addition to fraction 1 of the capsular antigen, also the antigen VW, Burrows and Bacon have discovered other characteristics which are specific to these strains and whose total or partial absence causes the attenuation of their degree of virulence and has a repercussion on their immunogenic potential. Jackson and Burrows [79] had brought to light the capacity of virulent strains to give pigmented colonies (P) on a synthetic medium containing hemin. Burrows and Bacon [17] took into consideration the possibility that these virulent strains may grow in the absence of purine (Pu+) or after the addition of purine (Pu-). On the basis of these facts, a strain of *P. pestis* that is virulent (in the pathogenic sense) both in the guinea pig and the mouse is constituted according to the formula F 1 + VW + P + Pu+. If VW is absent in this complex, the strain loses all virulence for the two species despite the possession of the other three characteristics. The loss of P+ is accompanied by the disappearance of virulence for the guinea pig and its attenuation for the mouse. The absence of F 1+ does not affect the virulence for the mouse but it does reduce it in the case of the guinea pig. Finally, the strains which

require the addition of purine for development are avirulent for both species.

We shall not discuss here the conclusions drawn by Burrows and Bacon from their studies on the experimental immunity in plague; we shall only mention that they found this problem very difficult to elucidate when realizing the complexity of the interaction between the factors involved, namely, the animal species used, the method of immunization and the nature of the virulence test. Mice, guinea pigs and rabbits differ in their response to the production of antibodies with live vaccines, and in each species the reaction to an efficient antigen is modified by the presence of a second determinant antigenic element of the vaccine.

We shall abstain from spending too much time on this subject; we shall discuss only what is of special interest for our topic under discussion.

With regard to the symbols inherent in a virulent strain, EV corresponds to the formula F 1+, VW+, P-. As for Tjiwidej, which is devoid of VW+ as we have mentioned above, it is symbolized by F 1+, VW-, P+. With respect to the characteristic Pu+ or Pu-, it is subject to such variations for the colonies of a given strain that we shall neglect it here. Moreover, our colleagues at Porton would consider that this characteristic has only an indirect effect on the immunogenic power, especially in the guinea pig, by producing a protective antigen which is not formed during in vitro growth by the Pu+ strains or in vivo by the Pu- strains. However, in the case of the latter, a repeat dose produces a high concentration of protective antibodies. Thus, when guinea pigs are administered two doses of a live avirulent strain F 1+ Pu-, their immunity increases considerably, and is comparable to that which is conferred by a single (or two) doses of an F 1+ Pu+ strain. Nothing like this is observed in the mouse. In our correspondence with Burrows we learned that strain EV was partially dependent on the addition of purine (uracil), let us say Pu± in a synthetic medium.

To sum up, the disappearance of the pathogenic power of the EV and Tjiwidej strains seems to be attributable to the loss of the characteristic P+ for the former and of VW+ for the latter.

Although we remain convinced that the superiority of the EV virus-vaccine in the immunization of the guinea pig is linked with the persistence of antigen VW, this view is not shared by the two scientists who have demonstrated it, nor by Chen et al. [23] who consider that antigen VW plays only a subordinate role in the immunity which, for them, always depends on the amount of fraction 1 in the vaccine, the fraction which is responsible for the production of protective antibodies.

It is from this different point of view that we consider the role played by antigen VW in the EV virus-vaccine, as we shall now discuss.

Antigen VW and Resistance to Phagocytosis

Let us stress first of all that this antigen with which we shall deal throughout this paper has not been isolated as yet, at least as far as we know. Its existence has been proved only by the presence of two precipitation lines which are consistently obtained by the techniques of Oudin-Ouchterlony in the virulent strains and the EV strain, and which are preserved by the antisera prepared by means of these strains after saturation with avirulent suspension. The precipitation-diffusion method in agar has been widely employed for the study of the antigenic structure of *P. pestis*, and the number of components determined in this way becomes as high as 18 for certain authors such as Lawton et al. [93]; moreover, it varies as a function of the composition of the culture media, the kind of medium (solid or liquid), the temperature of incubation (26 or 37°), and the pH. However, passive immunization tests in the mouse, undertaken in an attempt to discover, of this multitude of antigens, those which have a protective value, the corresponding antibodies of which are detected in the serum of vaccinated and protected animals, have not been conclusive. Already in 1955 Ransom et al. [117], who were the first to employ a technique inspired by that of Oudin, obtained results which attested an antigenic complexity which up to then had been unsuspected in the case of *P. pestis*, without being able to differentiate, in vitro, between a virulent and an avirulent strain of this pathogen. In fact, only fraction 1 and the toxin were isolated, since their chemical composition was known and their antigenic properties proven.

In support of his thesis, we shall give here the literal translation of a passage from a letter from Burrows, to whom we had sent a subculture of EV strain, received from the Belgian Congo, for control, and whose protective power in the guinea pig had sharply declined compared with that of the strain maintained in our laboratory. We were interested in knowing whether the sample in question still possessed the antigen VW. The reply was affirmative, and our colleague added: "I consider it not too likely that VW is actually the guinea-pig antigen, and I will demonstrate this in a forthcoming publication*. For example, I have obtained from a highly virulent original strain other strains which are F 1- VW- and nonpigmented, i.e., which have lost these three factors responsible for the virulence,

*This reference is to the paper which we have already mentioned [17], and which was published a few weeks after receipt of this letter.

but which nevertheless have preserved their power to immunize guinea pigs (but not mice) against a severe virulence test. From this I have concluded that an antigen other than F 1 or VW, or the latter combined with pigmentation, may play an important role in the immunization of guinea pigs." With certain reservations as to the nature of the test to which the animals protected by these F 1- VW- mutants had been subjected and the duration of their immunity, it is obvious that results of this type formally contradict those of the above-mentioned American authors, to be sure only as far as the guinea pig is concerned, since the mouse is not protected by these F 1- vaccines. In this respect, we may recall with S. Rowland [127], Boquet and Dujardin-Beaumetz [10] that *Pasteurella pseudotuberculosis* (abbreviated PST) which, as we well know, has many antigenic characteristics in common with *P. pestis*, immunizes the guinea pig rather well, especially if one uses an attenuated live strain; we have verified this at Tananarive [61a], although we did mention that with the strains employed the degree of protection was lower than that conferred by *P. pestis*.

More recently Thal [143] has shown that a strain of PST (32 IV), in the form of a virus-vaccine, conferred a strong immunity on the guinea pig. Now, while the two Pasteurellae are related by antigenic links, PST which is devoid of capsule does not possess fraction 1. This is a rather unexpected fact; Burrows and Bacon [18] have discovered, still by means of precipitation-diffusion, the presence of antigen VW in 27 out of 38 strains of PST isolated a short time previously from various animal species, but the question raised by this discovery is more complex than that of *P. pestis*. In effect, while the virulence of PST for the mouse requires the presence of factor VW, the virulence for the guinea pig is independent of this factor [19]. Since, according to the same authors, this antigen runs the risk of no longer being encountered after a few subcultures of strains whose virulence nevertheless remains quite marked, and since the live avirulent and protective strains had been isolated a long time ago and subcultured many times, it is improbable that this antigen plays a role in the PST-induced immunity against plague. Hence, it is possible that antigens other than F 1 and VW are wholly or partly responsible for the protection induced in the guinea pig by the two microbial species. The results of the precipitation-diffusion test are valuable; they attest the complexity of the antigenic structure of *P. pestis* and of PST without in any way solving the problem raised by their immunological relationships, since PST provides protection both against plague and pseudotuberculosis, while *P. pestis* confers only a homologous immunity.

Without prejudging the nature of this antigen VW, we have said above that we considered it as the dominant element among the factors which are responsible for the virulence of

P. pestis, because the resistance of this microorganism to the phagocytosis which governs the evolution of the infection is intimately linked with it. Thus, the EV strain which possesses this antigen behaves like a strain resistant to phagocytosis, as has been observed by Burrows and Bacon. Although the English and American scientists consider that VW does not intervene directly as an immunogenic factor, for us it nevertheless plays a great role in the immunization by the EV strain which, of all the attenuated and protective strains, has for a long time shown itself to be of the sole type VW+. It is to this characteristic -- as we have stated above -- that we must attribute the temporary proliferation of the virus in the animal organism where its vitality is preserved longer than that of the VW- germs; thus, as a result of a kind of transitory precautionary measure, an immunitary process is established which tends to duplicate that of a minor natural infection. Better than in an in vitro culture, the virus is placed in vivo, within the tissues, into ideal conditions of medium, temperature, pH, in order to produce there the antigens which lead to the formation of the protective antibodies and notably of fraction 1.

The interpretation given to the action of factor VW does not seem to invalidate the truth of the opinion expressed by the above-mentioned authors: it is to the in vivo production of fraction 1 from the capsular substance that the EV strain seems to owe its incontestable immunizing character. The prolongation of this manufacture due to the persistence of the virus in the organs supposedly makes up for the relatively low F 1 concentration of the in vitro culture suspensions, if we compare it with that of other VW- strains which, however, are far from conferring the same degree of protection on the guinea pig. This point of view expressed by K. F. Meyer during a conversation which took place some years ago is probably not unlike that of Strong and Otten to which we have referred in an earlier chapter.

It is permissible to reduce the importance of factor VW in the profile of F 1 by stating that this antigen also has the effect of partially inhibiting phagocytosis. Chen and Meyer [24] have noted this phenomenon when adding this proteinic fraction to normal rabbit blood; subsequently, Smith et al. [136], who tested the soluble portion of the ultrasonic extract of virulent plague bacilli, also attributed to F 1 the antiphagocytic activity of this extract which, however, contains the antigen VW. Even though this constituent of all virulent strains (and of strain EV), is linked to the resistance to phagocytosis, it does not seem to be its determinant element which can probably be attributed to F 1 present in both the VW+ and VW- protective strains.

In this debate which leaves many obscure points unexplained, we shall limit ourselves to drawing an argument from

an experimental fact to which our colleagues may perhaps not have devoted sufficient attention. This argument offers convincing proof of the fundamental difference which exists, with regard to the antiphagocytic potential, between the virus-vaccines F 1+VW+ and F 1+VW-. We have in mind the influence exerted by cortisone on the behavior of white mice inoculated with one or the other of the two types of antigens.

In 1955, Payne et al. [111] reported that the administration of cortisone to mice prior to the inoculation of live vaccines could, with certain strains such as EV 76 and F.7793-10, lead to a much higher mortality than in mice which did not receive this hormone; these mortality rates were in direct proportion to the cortisone dose injected between the limits of 0.05 mg and 2.5 mg per mouse in the form of a single intramuscular injection applied four hours prior to the vaccination. The death was accompanied by plague bacteremia and by marked lesions of the liver and the spleen. Normally, we know that with at least one of these strains (the best known, EV) the hepatosplenic reactions are transitory, as is also the case with the proliferation of the etiological agent which is only rarely encountered after one week, even if the mice succumb to intoxication or to an intercurrent affection; it is also necessary that the dose administered attain or exceed 200,000 germs. Under the effect of cortisone, it was sufficient to inoculate 10,000 cells -- although the inoculation in this case had to be applied peritoneally -- to observe this phenomenon; thus, in one of the authors' reports, we find that out of three lots of 20 mice, only two mice survived among those which had received 2.5 mg of cortisone prior to the vaccine, 18 mice survived among those which had been vaccinated without cortisone and 19 among those which had received the hormone alone. Now, with the A.1122 and Tjiwidej strains, which are avirulent and protective, cortisone caused no abnormal mortality under identical experimental conditions.

It goes without saying that we have attempted to verify these experiments on the same strains, doing so by scrupulously following the same experimental procedure; on the whole we did confirm the results of our colleagues from San Francisco [63a]. Nevertheless, the latter believed that the plague bacilli obtained from mice by hemoculture are capable, after several passages, of recovering their virulence, which was supposedly indicated by the fact that they had resisted phagocytosis. Experiments have invalidated this hypothesis. We have carried out ten successive passages of our EV strain in the peritoneum of mice which had been treated with cortisone, and investigated the pathogenic power of each subculture isolated from the blood. Against our expectation, we have had to increase, after the first passages, the dose of germs inoculated, in order to maintain the mortality rate of mice to which

the hormone had been administered at its initial level. After the tenth operation the strain was subcutaneously inoculated in a large dose into a lot of 40 new mice, while an identical number was given the same dose of a subculture of the normal EV strain. No difference was recorded as to the reactions of the animals of the two groups; when 20 days later they were tested for the degree of protection attained, the results were similar in both groups. This finding is contrary to that generally made when a serial passage of *P. pestis* strains, whose fading virulence one would like to restore, is carried out in mice or guinea pigs. In addition we have noted, analogously to Payne et al., that cortisone caused the emergence of an associated flora, Gram+ and Gram-, which forced us to carry out multiple isolations in order to isolate pure colonies of EV in the above-mentioned mice, even when the latter were sacrificed in the diseased state in order to prevent the proliferation of the germs of the associated flora. We know that when *P. pestis* finds itself in competition with these microorganisms in a culture or in the tissues, its virulence and its vitality are rapidly decreased. This microbial association, which is the rule in mice treated with cortisone, ought to prevent a real increase of the virulence of our strain. The mortality of the mice was certainly attributable to the vaccinal strain, but it followed a cortisone-induced failure of the phagocytic power of the reticuloendothelial system, which by now is a classic notion [104]. From our observations we drew the conclusion that cortisone should be absolutely ruled out in the treatment of human plague.

Thus, in these experiments EV behaved like a virulent strain. In effect there could not be any question of a toxic mortality favored by the hormone, because in that case one would have had to observe it also in the case of the A.1122 and Tjiwidej strains which are no less toxic than EV; in addition, some mice previously immunized by fraction 1 or by EV virus-vaccine itself resisted the test with cortisone + EV.

This was the state of our research when Burrows and Bacon published their first reports which brought to light the factors which control the virulence of *P. pestis*. We then wondered whether the presence of antigen VW did not account for the phenomenon observed in the mouse treated with cortisone. However, in view of the saying *"testis unus, testis nullus,"* we had to search among the strains of attenuated and protective virulence whether there were some additional ones which, like EV, were VW+. There were two strains which satisfied this requirement: one, F.7793-10 which had already been known as one endowed with a high immunizing power for the mouse, which we have already mentioned [146], and which Payne et al. had classified, with EV, as a pathogen for the mouse treated with cortisone; and the other, Harbin strain, which had been studied by

Otten some time ago and which he regarded as virulent in 1936 [108], then as avirulent two years later, and which was highly protective for the guinea pig -- analogously to EV -- contrary to his Tjiwidej strain [107]; we maintained this strain in our laboratory. Strain F.7793-10 was kindly sent to us by K. F. Meyer, and we noted that it protected the guinea pig just as efficiently as EV. The situation was the same with the Harbin strain which also produced an unusually high mortality rate in cortisone-treated mice. Doctor Burrows, to whose competence we addressed ourselves in order to learn whether these two strains were VW+, replied in the affirmative. Thus, there was indeed a correlation between the existence of factor VW and the apparent increase of the virulence for mice which were under the effect of cortisone, while nothing of the sort took place with the VW- strains.

Kozakevich et al. [91] have later confirmed the role of cortisone in the weakening of the resistance of susliks to inoculation with the EV strain. They noted, in addition, that the hormone considerably increased the sensitivity of these rodents to a virulent infection. This fact was exploited by Shtel'man [134] for the detection of a latent natural infection in rodents. He has, in fact, reported after experiments on *Meriones meridianus*, a species of the genus *Gerbillinae*, that it was possible to demonstrate *P. pestis* in the liver or spleen of animals surviving the infection and whose autopsy, after sacrifice on the 12th day, did not reveal any lesion when their ground tissue homogenates were injected into mice previously treated with cortisone.

Note. Cortisone in no way modifies the mode of reaction of the guinea pig to strain EV. Thus, of ten guinea pigs inoculated intraperitoneally with 500,000,000 live germs, five of whom had received 5 cg of cortisone intramuscularly four hours previously, two died from each group between the 5th and 9th day with the usual reactional splenic lesions without bacteriemia; the three survivors from each group, tested one month later according to our technique, were perfectly immunized (TI).

Parallel to the effect of cortisone, iron exerts the same type of action in mice inoculated intraperitoneally with rather small doses of EV which are supported by the controls without any harm. This was demonstrated by Jackson and Burrows [89]. Following these authors, we, too, have obtained the same results (unpublished); 0.2 ml of a filtered solution of 0.1 percent iron sulfate (or 40 µg of iron), injected into the peritoneum simultaneously with the microbial suspension (about 50,000 germs) causes a high mortality rate, while at this dose the iron alone is perfectly tolerated. On autopsy of the mice the findings were similar to those shown by the mice treated with cortisone. From this, Jackson and Burrows inferred that iron increases the virulence of the EV strain in vivo, while it

has no effect on other avirulent and protective strains such as A.1122 and Tjiwidej. Now the latter strain is P+, i.e., it utilizes iron in vivo in order to yield pigmented colonies, contrary to the EV strain. Hence, it must metabolize the iron also in vivo, and still its virulence is not modified. Do we not have sufficient grounds to attribute the indifference of these two strains to the administration of iron in vivo to the absence of the VW factor in these strains? Contrary to the interpretation of Jackson and Burrows, who believe that the EV metabolizes the iron in vivo and not in vitro, as a result of which an increase of its virulence takes place, we believe rather in the existence of a process whose course would be analogous to that caused by cortisone: decrease of the phagocytic action under the effect of iron whose affinity for the RES is well known; in this way, the latter would undergo a relative blockade. Let us add that, according to Jackson and Burrows, no metallic salt other than an iron salt is active under the indicated experimental conditions.

Finally, a last characteristic brought to light more recently tends to classify EV among the virulent strains. Higuchi and Smith [78] showed that on an agar medium with magnesium oxalate, only the avirulent elements of *P. pestis* multiplied at 37°; in effect, at this temperature the virulent organisms requre the presence of calcium. Higuchi and Smith have seeded their medium with highly diluted solutions (about 100 cells) prepared with 12 strains -- of them, six virulent and six avirulent -- comprising notably the strains EV, A.1122 and Tjiwidej. An identical seeding was carried out on blood-agar for control. None of the virulent strains yielded colonies on the magnesium oxalate agar, while on the blood-agar their number ranged from 18 to 110. The avirulent strains gave a number of colonies which was more or less the same on both media, with the exception of EV, which behaved like a fully virulent strain: 0 colony on oxalated medium, 20 on the blood-agar. While A.1122 and Tjiwidej are present among the six avirulent strains, Harbin and F.7793-10 are absent. We hope that this gap will be filled in order to prove, if necessary, the predominance of FW+ among the factors which determine the virulence of *P. pestis*, and especially its degree of resistance to phagocytosis.

In short, it follows that several facts converge in attributing to EV strain an incontestable degree of virulence, and we can understand perfectly well why Lawton et al. [93], who classify the plague strains simply into two categories, virulent and avirulent, place EV after the former and immediately before the latter, by writing "avirulent?." This question mark reflects the authors' scruples in regard to classying a microorganism virulent (pathogenic), when several million inoculations have been carried out with this agent in humans without the least accident or even a serious incident.

In truth, would it not be more logical to reserve to the strains of attenuated virulence a place intermediate between the truly virulent and avirulent ones, keeping in mind the definitions which we have recalled above? Burrows, as we have seen, has been led to adopt this concept [12] on the basis of the modes of reaction of the mouse and guinea pig to the inoculation of these strains. In this regard, the animal experiments and the antigenic analyses showing the presence of factor VW are in agreement in giving the EV, Harbin and F.7793-10 strains a privileged rank in the category of protective strains of attenuated virulence.

Reticuloendothelial System (RES) and EV Virus-Vaccine

The importance of the role played by the RES in the immunitary process whose culmination is the increase of the phagocytic potential toward *P. pestis* of the elements of this system (Fig. 4) has been demonstrated by Bablet with the EV strain [6], and then later by Meyer in a broader perspective [101]. Bablet has expressly underlined that the splenic reactions which are so manifest in the case of the live EV strain in the guinea pig are nonexistent with a killed vaccine.

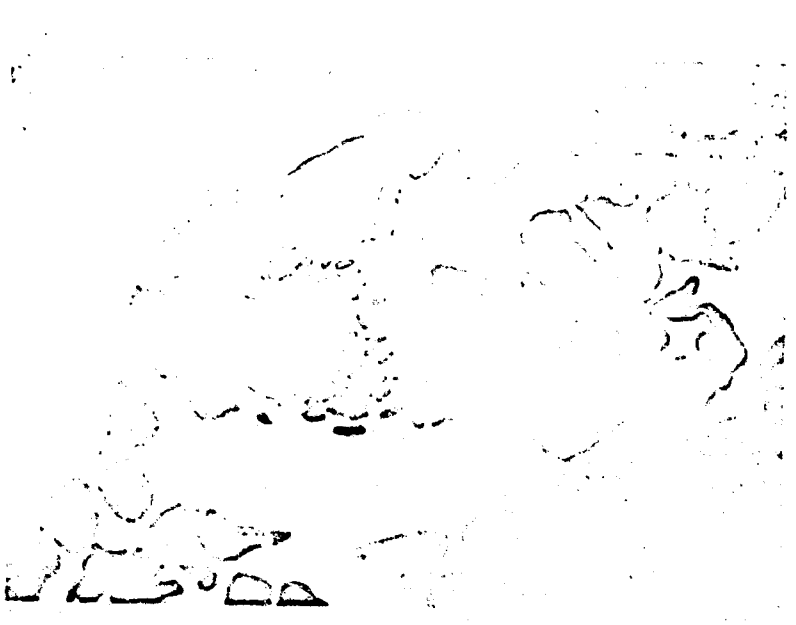


Fig. 4. Film Prepared by Impression of a Mouse Lung 7 Hours After Intranasal Instillation of Avirulent Strain EV 76. Note phagocytosis by the reticuloendothelial cells. (Giemsa, x 1500) (according to K. F. Meyer).

The work of the Russian authors points to the same direction. We have already mentioned in the first part of this article how much Korobkhova and Krainova were interested in vaccination by live germs, and notably in the use of our virus-vaccine in the immunization against experimental pneumonic plague [88]. We have also cited their colleagues Pokrovskaya and Kaganova who had administered the virus-vaccine to volunteers by inhalation, without any incident, after having subjected themselves to this experiment. However, this audacious experiment was only the practical confirmation of a thorough study of the mechanism of antiplague immunity in the guinea pig, which convinced these authors of the innocuousness and effectiveness of this mode of immunization [113]. In the first place, they noted (following Bablet with whose publications they were unfamiliar) that live vaccines produce a marked reaction of the macrophages which are produced neither by the killed vaccines nor the live strains whose protective power is zero or insignificant. In the protoplasm of the macrophage of an immunized organism, the bipolar elements "dissolve like sugar in water" (sic). Following this phagocytic process the macrophage remains intact, in contrast to that of the non-immune organisms. Pokrovskaya and Kaganova add in this connection that one frequently fails to demonstrate the presence of an immunizing substance in the blood of these animals, a phenomenon which they express in the following imaginative manner: "In the blood of plague patients, these 'antibodies' are found to be enclosed in the cells like a valuable liqueur in its bottle, so that, in order to obtain a small amount of the contents, it is necessary to break the vessel, in this case the cell."

However, it is the histological tests carried out during the process which sets in in the lung when this organ is attacked that have particularly captured these authors' attention. According to them, the lung is in an unfavored position with respect to the distribution of the histiocytes when compared with the spleen or the liver; this, in their opinion, is the reason which accounts for its low resistance and the difficulty encountered in the immunization against pneumonic plague. The subcutaneous way of administration allegedly fails to remedy this histiocyte deficiency of the lungs and, when the latter are affected, the organism may succumb despite the fact that it had been appropriately vaccined with a virus-vaccine. Above we have mentioned this problem of the immunization of the guinea pig against experimental plague pneumonia with live EV administered subcutaneously or by an ocular route, and noted the resistance of this animal which was confirmed about five years ago in our laboratory, but it is nevertheless true that some failures have been recorded. To remedy these failures, Pokrovskaya and Kaganova have attempted to increase the activity of

the pulmonary RES by means of repeated inhalations of live vaccine, using the EV and AMP strains with similar results. In this area these authors depend on Besredka's authority in the field of local immunization. Let us sum up their conclusions: In the guinea pigs which had received the vaccine subcutaneously and by inhalation and then tested by nasal inhalation of virulent germs, the survival rate was 80 percent compared with 60 percent after vaccination by the subcutaneous route alone; the mortality of the controls was 100 percent. The histological examinations have shown that by renewed inhalation, the mesenchymatous elements are stimulated, and a histiocytosis develops in the lungs, while efficacious polynuclear leukocytes make their appearance and contribute toward increasing the number of cells which participate in their protection.

The subsequent immunity permits the lung to combat the plague infection with a degree of success equal to that of any other organ.

Without having carried out a comparable cytological study, Meyer undertook an experiment whose results confirm the well-foundedness of the conclusions formulated by the Russian authors; this is worth mentioning here because the animals were immunized with the EV virus-vaccine; guinea pigs vaccinated by means of intranasal instillation (175,000 germs) were tested 21 days later by making them inhale a virulent suspension; by inoculation, the number of microorganisms reaching the lungs was counted at times 0 and showed, on the average, 1,025 germs in the non-immune animals and 925 in the immune ones. Four hours later, the number of viable bacilli was reduced in both groups to 1/10 of the initial value of the inoculum; in this stage the phagocytosis operated indistinctly as in the case of a subcutaneous infection. The multiplication increased between the 12th and 24th hours, and then progressed without hindrance in the controls, while the vaccinated animals showed the onset of a rapid and total sterilization of the lungs; after 24 hours, the former group gave a germ count of 24,000, the latter group, 13, and after 72 hours, the first group 62 million and the second group, 0.

The primordial role played by the spleen in the proliferation of reticular and endothelial elements during the immunization by the EV virus-vaccine, confirmed in the guinea pig by Bablet, does not seem to be of any lesser importance in mice, according to the histological tests of J. Levaditi (personal communication). Jawetz and Meyer [81] have confirmed Bablet's histological data in mice, using the avirulent protective strain A.1122. Hence, we have considered it of interest to compare the reactions of two groups of mice to the vaccine: normal mice and those which had previously undergone splenectomy and had completely recovered from the surgical

shock. We presumed that the ablation of the spleen would reduce the possibilities of immunization; the experiment confirmed this hypothesis [63]; the EV virus-vaccine, given in a large dose, is less well supported by the mouse deprived of the spleen than by the normal mouse. In addition, the splenectomy impedes the immunitary process initiated by the subcutaneous inoculation of the vaccine, as is shown by the following mortality rates after the virulence test: 50 percent in the vaccinated splenectomized lot, 10 percent in the vaccinated control lot, and 100 percent in the non-vaccinated controls.

Note. While the existence of three varities of *P. pestis* which differ from each other with respect to certain stable biochemical characteristics and especially with respect to their particular geographic distribution is now accepted, the modes of human infection are independent of the variety causing it; from the physiopathological point of view, the existence of Yersin's bacillus in one form only has not been challenged [64].

Apparently no differences in antigenic structure have been demonstrated up to now for the three types, called "orientalis, antiqua and mediaevelas" (Devignat), at least as far as the major factors -- F 1, VW and toxin -- are concerned. As for the EV virus-vaccine which derives from an "oriental" strain as do all others which have been imported by a sea route during the modern pandemia, its immunizing power is the same in all experimental infections produced in the guinea pig or the mouse by any one of the three varieties mentioned above [65].

Behavior of the Guinea Pig and the Mouse Inoculated with EV Virus-Vaccine by the Cerebral Route

Because of the neurotropism of the plague toxin whose action is exerted essentially on the central nervous system, we had thought of carrying out serial passages via guinea-pig brain in order to obtain cultures whose increased toxicity would, eventually, permit the preparation of a "formolated" toxin of the anatoxin type, since the behavior of the plague poison of proteinic constitution toward formol and heat is that of an exotoxin. Both in the case of the EV strain and of a virulent strain this objective was attained only after 12 passages. On his part, our colleague J. Bablet carried out the histological examination of the brain of guinea pigs which did not resist these inoculations. The results of these experiments carried out in 1942-1943 have never been published; below we shall sum up the conclusions of these tests, which fit into the framework of our topic:

a) A dose of 300,000 live germs (EV) leads to the death of the guinea pig within 40 to 72 hours, with lesions analogous

to those of the animals which receive approximately 1000 virulent organisms: thickening and congestion of the highly infiltrated meninges; numerous polynuclear leukocytes in the ventricles and the choroid plexuses; microabscess in the parenchyma in the vicinity of the ventricles. In both groups of animals the etiological agent, which does not proliferate in the cerebral tissue as it does in the spleen or liver of an animal which has died of acute plague, is always recoverable by inoculation of the brain. However, the blood, spleen, and liver are invaded only by the virulent germ, never by EV, and furthermore this invasion is not consistent; it is of moderate intensity and is detected by culture. The evolution of the process was much too rapid to permit the development of classic hepatic and splenic lesions; for Bablet, the death was attributable to the intoxication rather than to the infection.

b) Guinea pigs suitably vaccinated and then subjected, without damage, to the virulence test which reinforces their immunity, possess only a limited resistance to the inoculation of EV in the brain. They sometimes die only after several weeks, after having developed vast cerebral abscesses in which seeding still permits the isolation of the coccus bacillus. Bablet regarded this slow evolution and these delapidated states which are nevertheless compatible with an extended survival period, as having a certain analogy with his prior observations in the rabbit which he had inoculated with BCG by the same route (personal communication).

More recently, J. Levaditi has resumed his experiments of the same type on the mouse, by including these tests in an overall study of experimental virulence or avirulence on the basis of the data of histopathological tests [95]. As far as *P. pestis* is concerned, he compared the effects of the cerebral inoculation of a virulent strain and of EV in new mice and in previously immunized mice. The overall conclusions which our colleague was kind to communicate to us prior to their publication stress that mice inoculated with EV support this test much more readily than those which receive a virulent germ, which causes death in 48 hours. After sacrificing the first group after 3, 5 or 7 days, a microbial proliferation is noted which is hardly less abundant than in the second group; however, while the germs are freely dispersed in all animals, the cerebral vessels and the cells of their endothelia are invaded only by the virulent germs, never by EV. All the mice present signs of an acute meningitis, but the septicemia which is the rule in the case of the virulent strain is absent with EV. Another difference has also been noted: an afflux of polynuclear cells in the case of EV, and their absence in the case of the virulent strain. On about the fifth day, this polynuclear reaction (suppurative meningitis) is still present, with rarefaction of EV whose elements are no longer visible on the seventh day.

When the experiment was carried out with the virulent strain on mice solidly immunized with EV and the immunity increased by a subcutaneous virulent inoculation, the strain behaves like the EV strain and would be considered as partially or completely avirulent.

In conclusion, it must be admitted that the antiplague immunity is not always an absolute one, and that the brain cannot always be vaccinated. We understand why J. Levaditi asked the question: Do the physiological troubles which are caused by the cerebral test drown the immunity? As far as we are concerned, we regard this marked absence of cerebral immunity in animals which, moreover, are solidly immunized with the EV vaccine as an evidence of a correlation with the tardive meningic complications which have been reported in human plague which had apparently been cured by serotherapy (prior to the advent of antibiotics). The process develops insidiously during convalescence and leads to death within a few weeks or even months. Meyer et al. followed by others (quoted by Pollitzer [114]) have gathered together the most recent observations relating to this subject; these cases exhibit meningo-encephalitis lesions with abscess, in whose pus *P. pestis* may be identified. We could mention an (unpublished) personal case of ours, a patient suffering from bubonic plague who was treated at Tananarive with serum; after returning to the city, this patient died of a brain abscess which had been operated as an extreme measure after several months of neuropsychiatric manifestations and loss of vision. Although the circumstances did not permit us to take samples, the nature of these accidents does not seem to be in doubt, in view of their analogy with those reported by the preceding authors.

Homologous Interference Between the EV Strain and the Virulent Strain of *P. pestis*

The phenomenon which we have observed and described [66] without designating it by the term "interference" nevertheless responds to this designation. The experiments relating to this subject were based on our reflections regarding the heterogeneity of the cultures of *P. pestis*, which had already been recognized by Yersin [152] who considered this to be the reason for the dissociation taking place within the same microbial strain whose colonies may exhibit variable degrees of virulence. Otten [107] demonstrated the existence of this dissociation. We shall see later that this dissociation is accelerated under the influence of certain factors, and that it is important to try to prevent it if we want to preserve the characteristics of the EV strain as intact as possible.

With regard to Haffkine's lymph, we have stated [55] that the heating to 55° for 15 minutes of a virulent suspension

was capable not to destroy the vitality of all germs without, at the same time, causing an accident in the guinea pig which, moreover, was greatly benefited by the immunization. When we formerly noted that certain, but not all, guinea pigs died upon intraperitoneal inoculation of a large dose of virus-vaccine, we wondered whether a few highly virulent germs, which would be responsible for this mortality, did not subsist within the mass of avirulent or attenuated elements; but how could we prove this except by studying thousands of isolated colonies, something that is physically impossible? An increase of the virulence of the cultures of the peritoneal serosa, mentioned in our first reports [55a] led us in 1935 to isolate from our strain a single colony from which all the subcultures -- EV 76 -- are derived, subcultures which were used by us both in the laboratory and in human vaccination, and which had been requested from us, and where the figure 76 was sometimes followed by an "i" to distinguish them from EV 76 "t," a symbol for the "total" initial culture. Robic [122] had the idea, in 1941, to take a tube of this culture, which had been preserved in the refrigerator for 10 years, back to Tananarive and to study its behavior.

He succeeded in increasing its virulence by repeated passages in the guinea pig whose hemoculture furnished the subcultures required by this experiment. It is in this manner that, this time administered subcutaneously and not peritoneally, a relatively weak dose of the broth culture led to the death of the guinea pig within 6-12 days, and to the death of rats and mice in three days; the cause of death was septicemia. Since we, too, had this culture at our disposal we confirmed these results in Paris. We believe that an experimental artifice was responsible for the selection of virulent cells which were not detectable by our usual control tests. We have not had recourse to this artifice with culture 76 i, but it is not impossible that the conclusion would have been different. We were limited to the control of colonies isolated from blood or from the spleen of guinea pigs and mice sacrificed after the inoculation of massive doses of vaccine, or from the product resulting from the puncture of cutaneous nodules which developed after intradermal inoculation, and finally after hemoculture in cortisone-treated mice. We have failed to detect a colony which showed an abnormal virulence. The case was the same for colonies recovered after lyophilization. We then learned about a paper of Yegian and Budd [150], showing that the presence of a small number of virulent mutants in a strain of BCG could not, in all certainty, be detected by passage in the guinea pig. On the basis of this information we inoculated mice with a strong dose of EV virus-vaccine treated with 20-200 lethal doses of virulent *P. pestis*; later, other groups of mice were treated with vaccine and virulent suspension, these agents being separately administered either at the same time or with

an interval of 3-24 hours between the two inoculations, the virulent suspension always preceding the virus-vaccine. In five experiments with a total of 100 mice, using an equal number of controls, only one of which survived the virulent test, 67 resisted, and the latter, sacrificed three weeks later in a perfect state of health, showed no lesions other than a few cicatrices of small splenic nodules which are usually encountered after the inoculation of a heavy dose of virus-vaccine; no plague bacilli were detectable. If we substitute the live vaccine by an equal dose of EV killed by heat, the results are still significant but much worse than the preceding ones: there is a 28 percent survival rate, and 3 percent in the controls. However, when the virulent inoculation preceded that of the EV vaccine -- live or killed -- even by three hours the mice do not show the least resistance, and react like the controls.

This experimental balance sheet calls for attention. A priori, we were led to consider the existence of an accelerated evolution of the mixed infection, because we added to a severe virulence test the administration of a massive dose of germs possessing an attenuated virulence. We have discussed this hypothesis in [66]. It would seem that these germs led to the multiplication of the virulent ones which, by their nature, ought to develop first, with the consequences attested by the controls. It cannot be claimed that the process which led to the resistance of the mice subjected to the mixed inoculation involves a sort of camouflage or robbing of the virulent elements by the mass of avirulent elements; if things were that simple the latter, whether live or dead, would have given comparable results. However, the killed germs were found to be much less favorable than an equal number of live ones. Furthermore, it would not be possible to envisage a protection by specific antibodies whose production which, true enough, is quite fast in the case of the virus-vaccine, does not take place immediately. Perhaps the explanation of this paradox resides in a process of the same type as that reported by Landy and Pillemer [92], and Rowley [128] on the stimulation of the natural immunity in the mice after the administration of bacterial liposaccharides of diverse origin, as has been suggested to us by our colleague R. Laport, who is highly informed about everything pertaining to immunology.

To verify this hypothesis, we undertook a series of experiments the results of which were never published. We now have the opportunity of summarizing these experiments and our conclusions drawn from them.

1. We repeated the preceding experiments by the identical method, substituting the EV vaccine by the strain of *Salmonella typhi* 0.901, received from our colleague Le Minor, who assured us that this strain was completely innocuous when massive doses were subcutaneously inoculated into the mouse

(50 to 100 million organisms). While not as marked as with the EV strain, the results were nevertheless still significant: with the live 0.901 strain, 40 percent of the animals survived compared with 0 percent in the controls; however, when the Salmonella was killed by heating, not a single mouse was resistant to the infection, and the average time of onset of the infection was the same as in the controls*.

2. If the live 0.901 is administered 24 hours or more prior to the virulent suspension, the mortality is absolute. With live EV the case is quite different, and by spacing the two inoculations at 24, 48,.....-hour intervals up to 10 days, we made the following observations:

After 24 hours the survival rate, which was as high as 67 percent when the operations were carried out simultaneously, is only 42 percent; the percentage rises to 50 after 2 and 3 days, then to 70 percent on the fourth day, and attains the figure of 88 percent and stays there after the fifth day.

From these experimental data two concepts arise:

The first is that the presence of some virulent elements in a suspension of antiplague virus-vaccine such as EV whose density is not less than 500 millions of germs per ml cannot be detected by inoculation into the mouse, the rodent most receptive to plague infection. Hence, it is impossible to know whether the activity of the vaccine is due to the persistence of a small number of virulent cells or of mutants exhibiting this characteristic within an avirulent mass; nevertheless the fact of having studied a number of isolated colonies all of which seemed to us to possess a similar protective power would rather point to the inaccuracy of this hypothesis.

The second concept, which goes beyond the objectives which we had assigned to ourselves in the beginning, is the result of our most recent experiments; the latter tend to confirm the thesis of the above-mentioned authors regarding the stimulation of natural defense by a mechanism which is different from that resulting from the specific immunization and which exhibits a singular contrast even with the latter. In a single case, that of mixed inoculation, the Salmonella does not behave like EV, and the resistance of the mouse can be attributed only to an increase of its natural defense, because experience proves that strain 0.901 is devoid of all protective power against plague infection. However, with EV, this aspecific power only precedes that which causes the classical immunization which sets in rapidly; our results indicate that

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*A cholera vibrio which, when subcutaneously administered, is supported by the mouse in large doses, and which was used in an experiment in place of *S. typhi* 0.901, showed no effect on the evolution of the infection; the mortality rate was 100 percent, as in the controls.

the protection conferred after the administration of a heavy dose of vaccine manifests itself after the third day, and reaches its peak on the fifth.

In the practical domain, the superiority of a live microbe over a killed microbe is clearly reflected by the non-specific stimulation of the resistance of the mouse, as it takes place with the virus-vaccine EV from the point of view of the specific immunization.

The phenomenon of interference which we are discussing here takes place only under strictly limited conditions*. We do not have to refer here to the problem of bacterial interference in general, whether this interference is homologous or heterologous. Let us stress only that the acute infection analogous to the natural disease which the plague bacillus produces in the laboratory in sensitive rodents makes this microorganism eminently suitable for the study of a phenomenon whose mechanism is far from being clarified as yet.

Preservation of the Properties of Strain EV

In his letter which encouraged us to proceed in our studies along the path which we had chosen, Mr. Roux advised us, in addition, to seek out from among the other strains of plague bacilli which had become avirulent those which constituted good antigens and he added that "in this way you will have several possibilities. This requires an efficient bookkeeping."

This recommendation was a pertinent one; it was based on the first publications of Yersin and Carré [151] who had noted the variability of the antigenic value of the colonies originating from attenuated strains after a few months of preservation, and the loss of this quality after subculture simultaneously with the disappearance of the residual virulence with which their protective power was linked. While the term "dissociation" had not yet been fully accepted in our science, Yersin was nevertheless aware of this concept on the eve of its discovery. Mr. Roux had good reasons to believe that the EV strain would lose its immunizing power more or less rapidly, and since we had adopted the principle of vaccination by a virus-vaccine, we ought to have at our disposal certain substitute strains which possess the required characteristics. But, as we have stated, of several attenuated strains recognized to possess protective power, only the EV conferred a long-term immunity on the guinea pig, and we had to devote all our attention to preserving this strain with this dominant characteristic after it was decided to employ it in man.

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*An identical homologous interference was reported with *Pasteurella tularensis* by Eugene Skrodzki (*Bull. Soc. Path. Exot.*, Vol. 54, 1308-1314 (1961)).

The desired degree of attenuation was attained after five years of monthly subculture at 26-28°C; afterward, these subcultures were limited to two per year and the three-day subcultures were placed into the refrigerator and maintained there at around +4°. The sole medium employed was Martin broth agar at pH 7. While it was possible for the preparation of vaccine batches to have recourse to peptone agar when we were surprised to discover that the broth contained substances which inhibited the initial growth of weak inocula of *P. pestis* [67], we always preferred, as a preservation medium, Martin agar widely inoculated on the surface or at the bottom, some of the tubes being sealed and the others carefully capped in order to maintain a certain degree of humidity within.

Of the factors capable of modifying the qualities of strain EV in the sense of an attenuation, the temperature is no doubt the most important. The prescription of preserving the cultures in the refrigerator at a temperature of about +4° is very important. The proof for this was furnished by Robic [122] when, carrying out our first studies in this respect, he reported that a culture of EV, subcultured in parallel to EV 76 but preserved at laboratory temperature (about 20°C) had after three years practically lost its protective power as well as that of producing the characteristic splenic reactions in the guinea pig after inoculation of a heavy dose by the peritoneal route. Moreover, experience has taught us a long time ago something which was unknown at the end of the last century, namely, that whereas the vitality of a strain of *P. pestis* is only slowly influenced in a liquid preservation medium below 25°, the preservation of its virulence requires that it be kept in the refrigerator, a notion which has become classical in our specialty since then, but the lack of awareness of this fact was surely responsible for the rapid drops of virulence which Yersin had observed in his cultures exposed for part of the year to the tropical heat. Not less prejudicial are the abrupt temperature changes to which cultures sent to far-away places are exposed if no special precautions are taken. Before using airplanes to this end, and before it could be guaranteed that the package would be protected from great temperature changes between the time of its mailing by the sending laboratory and its arrival at the receiving laboratory, transportation by sea was certainly responsible for some of the surprises experienced by some researchers who had received the EV strain. We gave some examples of this at the beginning of the present paper, and we were even able to confirm in the case of cultures returned to us for control that a notable loss of the qualities of the strain had occurred, compared with the strain maintained in our laboratory. On his part, Otten has experienced a particularly instructive misfortune with his Tjiwidej strain [107]. After returning to Bandung, following a stay in Holland, he

resumed his studies by using the culture which he had taken from Europe and which he had preserved in deep agar without any special precautions. For the first time since the beginning of his studies (1930), he noted a loss of the antigenic power of his strain. He thought that this could be attributed to a dissociation of the colonies, some of which appeared to him morphologically atypical, but vaccines prepared with all these types were found to be devoid of any effectiveness. He then tested a sample which had been continuously preserved in Bandung in the refrigerator, and found that its original immunizing power had not undergone any change. Much has been written about the variations in the appearance of *P. pestis* colonies and Yersin had already pointed out that there were dwarfs and giants among them after the initial seeding of the material taken from man or animal; however, we know that these differences are not stable, as is shown by subsequent subculturing. Then there were the types R and S, which were described by analogy with microorganisms such as the enterobacteria, where these characteristics are accompanied by profound modifications of the antigenic structure. Without dwelling on this topic too long, we shall state only that the morphological appearance of the *P. pestis* colonies in no way prejudges their antigenic quality. It was even possible to abandon the expressions R and S and replace them by the labels "smooth" and "non-smooth," the latter, equivalent to R, being the attribute of the most virulent colonies. Now, in the case of the EV strain, we have observed all these types without being able to derive from them any valid data for the practical field of immunization. We shall see later on how it is possible to change from one type to the other when we discuss the studies carried out in order to regenerate or reinforce the eventually decreasing strengh of strain EV. However, in 1959 Wake [148] described another morphological variety -- the third colonial form -- discovered in all strains of *P. pestis* maintained on artificial media without animal passage, or simply stored for several months at 0-5°C, thus independently of their degree of virulence, their antigenic structure and their protective power. The culture medium employed was nitritive agar treated with 10 percent bovine blood. The author stressed that by their rapid growth, their size, their capacity and the degree of their whiteness, these colonies could be classified into R and S (or smooth and non-smooth) types. The cells have the form of cocci rather than coccus bacilli and, when subcultured in broth, render the medium uniformly turbid. Finally, this third variety was also isolated from the organism of immunized animals which had resisted a virulence test.

The importance which we attach to this work resides in the observations made by Wake on the EV strain and in the comments relating to these observations. Starting from an isolated colony of the smooth type, he carried out a series of

subcultures at 37°C from one singular colony to the next. At the 11th subculture, of 334 smooth colonies three were manifestly of the "third colonial form," and the cultures deriving from them had a toxicity that was six times weaker than that of the parent strain; fraction 1 of the capsular antigen was no longer detectable by precipitation-diffusion on agar. When virulent strains are subjected to an identical experimental procedure, this result is confirmed by the loss of virulence. The protective power of the suspensions of these live organisms is insignificant or zero. As far as the EV strain is concerned, the author considers it legitimate -- something which we have always maintained -- to link the protective value of our virus-vaccine to the permanence of a partial virulence and of the toxicity of the strain from which it originated. If it were necessary, the method used by Wake would prove how the dissociation of *P. pestis* is favored by a succession of subcultures, especially at 37°C, which exclude the maintenance of the cultures in the refrigerator in the interval. However, the author places this phenomenon into a much too narrow framework because, apart from his "third colonial form," the strains devoid of all antigenic power (e.g., Pecha) do not differ with respect to the appearance of their colonies from most others, except perhaps by a delay in growth and by lean cultures, in contrast to those of the type defined by the Japanese author which appear after the sixth hour. At the present time, we find it impossible to state what are the factors which, apart from the temperature, intervene in the maintenance or the loss of the integrity of the original characteristics of *P. pestis*. Our practice spanning a period of 30 years has convinced us that the mode of maintenance and preservation of the strain EV which we have adopted and perfected does not cause a deterioration in quality. Until now we have ruled out the use of lyophilization, despite the vogue which this technique is currently enjoying in microbiology, because of the uncertainties which this method would present for *P. pestis* in general and the EV strain in in particular. The following considerations justify our reservations.

If one's objective is only to preserve the vitality of the strain with its initial characteristics, then there is no reason or need to abandon a simple and convenient technique which has stood the test of time. The interest of lyophilization which is of a considerable advantage for microorganisms other than *P. pestis* would reside, for us, in the possibility of preparing a stable dried virus-vaccine which is not too sensitive to temperature variations and which, after re-suspension, would permit the immunization of man (which is the case for BCG); this would represent a major advance because it would remedy the great inconvenience of not being able to store a vaccine for more than 15 hours, so that in current practice

this vaccine must be prepared on the spot. A first question arose in this connection: would not the freezing followed by drying cause a dissociation which would take place in an unpredictable direction depending on the degree of virulence of the cells which have remained viable? From this angle we have not noted any anomalies and the cultures of several colonies recovered after lyophilization have conferred on the mouse a satisfactory protection and normal reactions. Working alone or with J. Courdurier, several batches of ampoules were prepared, and they have yielded rather divergent results with regard to the count of surviving germs. A few ampoules of this "dry" vaccine were sent in 1951 to Tananarive where Robic [123] successfully tested them after having stored them at +4° for 5-12 months; the count of live germs and the immunization of the guinea pigs gave the expected results. In volunteers who were inoculated with this vaccine, the latter produced the usual reactions. By contrast, a second shipment in 1952 [124] gave disappointing results. On this occasion our colleage made an important observation -- that the germs became fragile after resuspension, and their count dropped to 0 when the seeding was carried out after a few hours, which had never been observed before in the ampoules of vaccine preserved at +4°C, even when the storage period extended for several months. At that time [68] we stressed how long-lived the plague bacillus was when suspended in physiological saline solution, even if the latter was not buffered, and the use which we have made of this property in the elaboration of a method of detection of human plague before and after death. The lyophilization was probably responsible for this incident, but we were able to note a repetition of this event with a suspension originating from the subculture of an old, non-lyophilized culture. This is a new and important fact; it must be taken into account in the control of the strain because, if it became generalized, it would indicate a profound modification of its behavior.
K. F. Meyer of San Francisco had used lyophilization which he considered to be the ideal form of preservation. In 1949 he was surprised to note a change in the antigenic power of the strain; we had to send him a new culture and to advise him to cultivate it according to our technique. Meyer had also asked Pavarel [35] at Tananarive for his opinion, and the latter answered in the same vein. Quan et al. [116] have had satisfactory results by subjecting the strains A.1122 and EV 76 to slow drying after freezing of the suspensions in a solution composed of salts and lactose, these constituents being present in the same proportions as in skimmed milk. In truth, the problem of the lyophilization of *P. pestis* is not a simple one, and its study was undertaken by Heckly et al. [77]. It brought to light a few factors which affect the viability of the microorganism during and after the operations, and showed that

unawareness of these factors was at the root of the disappointments of those who had believed that the plague bacillus would not behave differently from most viruses or from bacteria in the application of the general principles of lyophilization. In view of these uncertainties, Mead et al. [98] adopted a new orientation and, as a result of work carried out recently, they recommend the preservation of *P. pestis* at -23°C in the simple frozen state, in a lactose-containing medium with pH 7.4 phosphate buffer in which the germs are suspended after centrifuging of the cultures. The arguments advanced in favor of this procedure, which makes it possible to maintain both the virulence and the viability of 50 percent of the cells for two years, would seem to legitimize its substitution to lyophilization, and for us it would be of interest for the preservation of the EV virus-vaccine. It is for this reason that we have mentioned it in this paper.

Three Special Cases of Dissociation of Strain EV

When in 1946 we performed a purity test on a virus-vaccine suspension by adding to a broth tube one drop of specific bacteriophage which temporarily inhibits the growth of *P. pestis*, we were surprised to note that the medium became strongly turbid after 48 hours. Our first assumption was that this was due to contamination, but this turned out to be incorrect; the bacillus had well preserved the morphology of the plague bacillus, it was gram-negative and immobile like the latter, but it had lost the antigenic and biochemical characteristics of the EV strain. On an agar tube of this culture left at room temperature for one month we noted a glassy formation which attested to a profound modification of the culture, which could no longer be subcultured; however, against this uniform background there appeared several new colonies which exhibited all the characteristics of the EV strain, including the sensitivity to the phage. We did not succeed in reproducing this phenomenon in 50 broth tubes seeded with the same sample of EV and by the addition of one drop of phage. Never again, during hundreds of control tests carried out in Madagascar or Paris, did we make a similar observation.

We decided to report this phenomenon only five years later [69] when we again had exposed to the action of the selected phage a brick-red mutant of an EV culture maintained without subculturing for ten years. The existence of pigmented colonies is not an exceptional event in the case of *P. pestis*, and the phenomenon would hardly have been of interest to us if the study of this mutant, which behaved like a saprophyte -- a very fragile saprophyte growing only between 15 and 25° -- had not shown the appearance, in a 16-day-old culture in which the majority of pigmented germs were dead, of

a culture typical of our EV strain with all its characteristics. In this case, too, our efforts to reproduce the phenomenon with the brick-red mutant remained fruitless.

The reason for publishing these facts is that they support a hypothesis formulated by Herelle some time ago regarding the possible reversibility of the mutants produced or selected by the bacteriophages in nature, and their incidence in the renewed outbreak of cholera epidemics in Bengal. In this way, Herelle, to whom we had communicated our observations on *P. pestis*, found a justification of his hypothesis which up to then had been completely gratuitous (personal communication). On the basis of this initial finding, we subsequently found new elements in favor of this hypothesis, when extending our experiments to virulent strains [70].

We owe the story of the third example of dissociation to Devignat [27]. In his case, the dissociation took place spontaneously. The phenomenon manifested itself in the agar culture boxes during the preparation of a lot of vaccine; ochre-yellow colonies of the S and R type appeared on the third day and were subjected to a complete study by the author. The salient fact emerging from this study is that while these "mutants" had preserved all the morphological, biochemical and biological properties of the EV strain, their immunizing power in the guinea pig had almost completely disappeared.

Increase and Stabilization of the Immunogenic Properties of the EV Strain

The regular controls to which the EV strain had been subjected in Tananarive and Paris indicate the concern of those responsible for the preparation of the virus-vaccine that they might be surprised to find, one day, that a sudden modification of its properties had taken place. Let us recall Yersin's express recommendation in this respect. Would this be a return to virulence? Under the conditions of subculture (26-28°C) and maintenance of the strain on nutrient agar from which we have never departed, such a development was highly improbable; moreover, no examples of it were known in the case of *P. pestis* cultured on the usual media. On the other hand, the anticipation of a total or partial loss of the protective power was legitimate; under several circumstances, we noted that this did in fact happen, without being able to determine the cause of this development. Apart from cases where our observations were made on cultures sent to us from abroad, so that we did not know how these cultures had been preserved and transported, we sometimes noted certain anomalies also in the case of samples which we, ourselves, had collected; thus, a 15-month-old culture maintained at +4° could no longer be subcultured despite the fact that the parent culture which was 2 or 3 years

old had preserved all its characteristics, including its vitality. These incidents remain inexplicable for us; we considered the possibility of a contamination by a phage, but have failed to detect a lytic principle in these cultures. On smears, the germs have a dot-like appearance; they are weakly stained with fuchsin while an old but still live culture exhibits cells which stand out sharply with their morphology and color against a background of disintegrated elements. Hence, it is prudent to have substitute cultures available by setting aside and preserving, after each subculture, a few tubes which are sealed and maintained in the refrigerator, as a precaution against at least one risk: that of losing the strain.

In view of these observations, we had no illusions in believing that the properties of our EV strain were immutable, despite the fact that the procedure empirically adopted for its preservation had given full satisfaction, especially at a time when the vaccination of humans was our predominant concern. Hence, in addition to numerous culture samples maintained as described above, we have collected others originating from animal passages -- guinea pig or mouse -- after seeding of ganglionic serosa, blood or splenic pulp during the period when we know that the term, if administered in a high dose, subsists live in the tissues. A few experiments carried out with these subcultures did not reveal any marked change in the virulence or immunizing power.

E. Korobkova [89], rightly considering that the stabilization of the properties of the EV strain was a crucial question for the manufacture of the virus-vaccine, carried out important studies aiming at ensuring its stabilization and even at increasing its immunogenic properties. A culture which had been preserved in the refrigerator for several years was subcultured on nitritive agar treated with glycine, then inoculated in the peritoneum of guinea pigs. When the animal succumbed to this test, subcultures were obtained from its blood, liver and spleen, which served for carrying our new peritoneal passages. After seven operations the virulence was not increased perceptibly. Blood, liver homogenates and spleen homogenates were suspended in physiological saline solution and placed into 0.5 ml ampoules which were then frozen and dried in vacuo. After a three-year stay in the refrigerator, the culture recovered from one of the ampoules protected the guinea pig much better than that of the initial sample which had not been passed through animals and which had lost a large part of its antigenic power. Korobkova stressed that the most active virus-vaccine originated from splenic pulp cultures and not from blood cultures; she considers blood as a not too favorable medium for the preservation of the EV strain.

On his part, Wake [149] reported that in *P. pestis* the S colonies which appear within the R colonies and which arrest

to an attenuation of the virulence and of the antigenic potential were remarkably stable in vitro. Mice had been subcutaneously administered a high dose of suspension consisting solely of germs originating from the S colonies of an old EV culture. After several passages, he was able to isolate typical R colonies from the tissues of mice which had died or were sacrificed during the 13-hour period immediately following this inoculation. It cannot be doubted that the passage through the receptive animal resulted in the selection of the germs that are most resistant to phagocytosis, hence which are the most virulent ones, and as far as the EV strain was concerned, it had the further effect of maintaining, and even increasing, its immunizing properties in case of failure. Furthermore, is this not the procedure commonly used in the laboratory for increasing the virulence of *P. pestis* when the latter had decreased?

Cavanaugh and Randall [21] gave an explanation for this by showing the role played by the monocytes in this process of the transformation of S-type germs (sensitive to phagocytosis by polynuclear leukocytes) into resistant forms -- R or M -- depending on whether or not they are encapsulated. These experiments were carried out on a virulent strain but their conclusions are no less valid for the EV strain, due to the latter's particular antigenic constitution. It can even be considered that, through a temporary multiplication of the germs in the monocytes of the spleen, colonies are obtained whose virulence scale would increase until attaining the original pathogenic power of the strain. It goes without saying that one cannot be careful enough when using an EV virus-vaccine regenerated in this way for human inoculation, despite the fact that the antibiotics presently at our disposal provide full safety in case of a serious incident. We were unable to develop our investigations in this area, and were only able, prior to leaving our Service, to leave for our successors in the laboratory an array of EV cultures for studies to be carried out on a more speculative than practical level, considering that the current world situation of plague relegates the problem of vaccination, which had been our predominant preoccupation, into the background.

It is not impossible, without resorting to animal passage, to consider the maintenance of the integrity of the characteristics of the strain by cultivating it in media other than agar or Martin's broth. Avanyan and Gubina [4] specify the addition of iron to the broth or nutrient agar; the iron is extensively utilized by *P. pestis*; this element increases the growth of the microorganism and increases its catalase content, as has been found by these authors, notably in the case of the EV strain. Now, Rochenmaker [126] had reported a long time ago that a correlation exists between the catalase content and virulence of *P. pestis*. Nevertheless, we do not find in the

work of the Russian authors any indication of an increased virulence of the EV cultures, but only an increase in the virulence of two other attenuated strains inoculated into the guinea pig after administration of iron sulfate. We have seen above that the mouse treated in this manner did not support the EV virus-vaccine, but that we attribute this fact to a partial blockade of the reticulo-hystiocytary system analogous to that which is carried out by means of cortisone with the attenuated VW+ strains, and not to a real intensification of virulence.

Culturing at 37° which activates the production of capsular antigens and particularly fraction 1 may also contribute to the prevention of the degradation of the strain. We have had in our hands a subculture received from K. F. Meyer maintained at 37° on blood-agar whose protective power for the mouse was greater than that of the culture of our laboratory; we were not surprised by this, because the F 1 content of the EV strain which was quite low originally, has to increase at 37°, and for the American authors this fraction represents the major, if not the sole, immunizing antigen. However, the other factors of pathogenic power may perhaps undergo an intensification of their action, if we take into account Favarel's observation [36] according to whom a virus-vaccine prepared with cultures incubated at 37° engenders local and general reactions which, notably, are more pronounced than those caused by the vaccine manufactured from cultures incubated at 26°C.

Finally, we shall end this chapter by describing an experimental study of Korobkova [90], who has sought to increase the effectiveness of the EV virus-vaccine not by acting on the strain itself but by perfecting the technique of vaccination; she envisaged a double vaccination, increasing the inoculated dose, and increasing the interval between the two inoculations, factors which the author considered the only way to ensure a solid and durable protection against all clinical forms of plague. The cutaneous vaccination (based on the role of nervous receptivity in immunogenesis (sic) was, in her opinion, more efficient than subcutaneous administration, since the reactions would be less intense and the contraindications decreased. These experiments were carried out in guinea pigs with a culture which was 50 percent successful when inoculated once and 100 percent successful when inoculated twice. It is undeniable that a second inoculation performed 2-3 weeks after the first reinforces the immunity, as we have seen in the case of the mouse where we were 100 percent successful, while the same percentage was attained in the guinea pig with a single dose. If we had had to apply this technique in our mass vaccinations in Madagascar, we would have been faced with insurmountable practical difficulties, because we had relied precisely on the advantage of a single inoculation which we

had considered sufficient on the basis of the data furnished by our experiments with guinea pigs. However, an annual revaccination performed when the circumstances called for it certainly resulted in increasing the protection conferred by the previous vaccination or vaccinations. As for the cutaneous administration of the vaccine, we knew from our first experiments that the guinea pig could be immunized by this route (scarifications), but that the subcutaneous route was preferable; from every point of view we consider that the subcutaneous route is also that which offers the maximum guarantees in collective vaccinations.

Various Studies on the EV Strain

Under this heading we collect a variety of studies relating to the EV strain, some of which are currently under way; although they have no immediate connection with immunology, we feel that they should be included in this paper.

Leshkovich [94], who studied the action of x-rays on the virulence and immunogenic power of *P. pestis* noted that 43 days after three irradiations, virulent strains had lost a part of their pathogenic power for the mouse and guinea pig. The exposure of the EV vaccinal strain under the same conditions as the virulent strains had not modified its immunizing properties; the author infers from this that x-rays have no destructive effect on these properties.

As part of their studies on the fate of *P. pestis* in the organism of several species of fleas, Cavanaugh et al. [20] inoculated heavy doses of avirulent strains into mice and in this way produced a transitory bacteremia. Six *Monopsylla anisus* were infected with EV, and it was observed that their proventricule was blocked. All the tests of the transmission of the infection by biting new mice have failed.

At the Pasteur Institute of Madagascar where the EV strain is constantly being studied in its various aspects, Brygoo and Courdurier [11a] have developed an effectiveness test on mice which, because of its great precision, usefully completes the test which we always performed on the guinea pig. This mouse test remains indicated for the control of the strain. Daod Nathoo, Dodin and Brygoo [25] undertook the complete antigenic study of EV by precipitation-diffusion in agar. The investigations currently under way already show the complexity of the reactions which vary as a function of the temperature of incubation of the cultures and also of the origin of the sera employed (horse, rabbit). The analysis of the total extract reveals 17 flocculation bands with an "antiplague immune serum globulin" (Lederle) prepared in the rabbit; the RNA and DNA extracts show 12 and 2 bands, respectively. The authors expect to carry out tests to determine the extent of protection of the

animal by the RNA and DNA extracts. The importance of these studies resides in the fact that they will, perhaps, shed some light on the nature of the immunizing antigens of which only Baker's fraction 1 has been identified, as soon as it is possible to obtain from strain EV a series of samples of more or less degraded antigenic value, and to carry out comparative analyses, from which it is legitimate to expect a certain clarification of the problem.

In the framework of the metabolism of *P. pestis*, Dodin and Brygoo have studied that of the EV strain and carried out, on nonproliferating suspensions, a quantitative study of the utilization of the sugars rhamnose and glucose [28], xylose, mannose, and glucosamine [29] The succinic acid dehydrogenase activity of a concentrated suspension of virus-vaccine as a function of the length of preservation at +4° showed that the enzymatic potential was preserved for a long time at this temperature, inasmuch as the reduction was only 50 percent after 90 days [30].

Finally, certain special globulins, which have a very alkaline isoelectric point and for the most part consist of antibodies, are characteristic of the antibacterial sera of the horse. Sandor [130] and others are now studying these globulines, especially in horses hyperimmunized by the EV strain; this strain has been found to be very powerful from the point of view of the production of these globulins.

Epilogue

The studies which we have discussed at length above give cause for reflection. A plague bacillus which in many ways is analogous to the virulent strains could, for the past 30 years, be inoculated in the live form with impunity into hundreds of thousands of persons. For authors who have not known the circumstances which had led to the use of the EV virus-vaccine and who, later on, carried out the analysis of the antigenic structure of this strain, it must have appeared that what we had done was rather rash. Certainly it was rash, but it had been preceded by years of animal experiments, which is the only concrete reality which permits drawing decisive conclusions in the field of immunization. Plague has the peculiarity of being a zoonosis whose pathological manifestations in their various aspects are identical in man and in sensitive animals, both in natural infection and in experimental infection. This represents an advantage compared with most human infectious diseases against which there exist vaccines whose protective value is generally evaluated only by the titer of the seral antibodies which, precisely in the case of plague, are often absent; when they are detectable, their relationship to the degree of immunity conferred by the disease or the vaccination

remains questionable. Was it not logical and in line with Pasteur's teachings to offer mankind the benefits of a virus-vaccine which was found to have an effectiveness incontestably superior to that of the killed vaccines? Nevertheless, if what we know today of the antigenic structure of the EV strain had been known at the time of our first studies, it is probable that we never would have dared to inoculate man, at least not on a large scale. It is fortunate that fate has settled this problem otherwise.

It is no less fortunate that eminent scientists have visited Madagascar to investigate, 17 years after the first applications, the nature of the reactions brought about in man by the EV virus-vaccine. An official mission comprising three highly qualified medical men from the United States -- Doctors Smadel, Goodner and Woodward -- arrived in Madagascar in 1951 to study the behavior of the plague endemia and the means of defense with which this disease was being confronted. They soon contacted the Pasteur Institute and it seems that they were primarily interested in the vaccination program, especially since at that time Dr. Robic was still in charge of the latter. He certainly complied with his guests' wishes by performing a vaccination session whose preparation was attended by his guests. A detailed report of this operation as well as the summary of and comments on the report written at the end of their mission by the American doctors was published by Robic [125]. We quote the essential passages of this report:

"We have vaccinated a detachment of parachutists, all of them young soldiers, consisting of Europeans recently arrived from France and Malagasy who had not yet been vaccinated; thus, they were persons vaccinated for the first time in whom we know that the reactions are generally more pronounced. The vaccine employed had been prepared and harvested on the eve of the vaccination and the suspension was immediately prepared in the presence of Dr. Smadel and his collaborators -- thus, we had a perfectly live vaccine -- and inoculated into ourselves and into two of our Malagasy technicians. Next day, after the control of bacteriological purity, the vaccine was inoculated into the soldiers; in some of them the forearm was used as the site of inoculation, in others the shoulder (infraspinous fossa). The American doctors attended this session, and Professor Goodner personally and attentively followed the behavior of the vaccinated persons, himself taking the temperatures, checking the inflammatory reactions at the site of inoculation, looking for ganglionic reactions and noting the general state of health of the vaccinated subjects. He could verify that all local or general reactions rapidly subsided."

Below we quote the conclusions of the report written by Dr. Smadel, translated verbatim by Robic:

"According to the description of the French workers and our own observations, the local and general reactions in persons who have received an injection of EV vaccine are a little more severe than those brought on by the classical antityphoid vaccine. They are more pronounced in Europeans, after subcutaneous injection in the lateroposterior surface of the forearm than after subcutaneous injection above the shoulder blade. The first route is used in the Malagasy* to avoid having to ask them to disrobe, and the second in Europeans. Nevertheless, the Malagasy are less sensitive because, after injection into the forearm, they have a less intense reaction than the Europeans inoculated in the shoulder. Intramuscular injection is prohibited because of the severity of the local reactions. On the other hand, the tests carried out in order to obtain a proof of the proliferation of the EV germs in human subjects gave negative results, despite the fact that under several conditions we found, after the inoculations, a few live germs at the injection site or at the level of the regional ganglionic chain (some data on this point may be found in the *Archives de l'Institute Pasteur* of Tananarive, years 1937 and 1938)." Robic emphasized that Dr. Smadel completely subscribed to our conclusions published in these *Archives* in 1938.

Nevertheless, the members of the mission were disconcerted about the reasons which have made it necessary in 1949 to give up the policy of massive vaccination campaigns and annual revaccination. We have replied to this in the first part of this work, and we shall quote in this connection a passage from a paper written by Robic: "Prophylaxis by massive vaccination has the advantage that it has stopped the spreading of plague on the High Plateaux at a time when no other effective method was known either in the area of prevention or in the area of therapy; there was no DDT, no sulfonamides, no streptomycin. At the present time, prophylaxis

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*The choice of the forearm for a subcutaneous vaccination may appear abnormal, but it was adopted for several reasons: ease of execution during the gatherings of thousands of persons of the two sexes and of all ages who did not have to disrobe; convenient control of the local reactions and of any epitrochlear and axillary ganglionic repercussions which may have developed. However, the Malagasy themselves, many of whom sleep on a mat placed on the ground or on a rudimentary stretcher complained of pains caused by the pressure of the ground or of the wood when they were vaccinated in the shoulder blade.

There is no doubt that the local reaction, which is more marked in the forearm than in the dorsal region, is connected with a slower absorption of the vaccine and with a longer persistence of the live germs in the cellular tissue, a fact which, in the final analysis, ought to be beneficial for the immunization.

aims at the eradication of plague through the destruction of the virus reservoir, the flea of the rat."

We have also stated why, between 1954 and 1958, the collective vaccinations were temporarily resumed. For the past three years all vaccinations have been suspended in Madagascar, and the number of cases of plague has in no way been affected by this interruption.

The era of mass vaccinations, regardless of the type of vaccine employed, is now over not only in Madagascar but all over the world. In effect, the present situation of plague relegates vaccination, which has for such a long time occupied a predominant place in the fight against plague, into the background. For the past 25 years, the incidence of human plague has been decreasing everywhere, and this decrease has been spectacular during the last decade, as we have stressed in our introduction. From the time when the prevention was deliberately oriented toward systematic disinfestation which took precedence over deratization, there has not been a single plague focus in the process of development that was not rapidly eradicated by the judicious use of contact insecticides, with DDT remaining the most important of these agents.

Parallel to the advantage of a better conceived etiological prevention, the prognosis of the disease which today is curable in all its clinical forms including the primary pneumonic form, has been radically changed through the advent of fungal antibiotics. Finally, according to the experience acquired in Madagascar, chemotherapy (sulfonamides) on its part, considerably reduces the risks incurred by persons who had been in contact with patients with pneumonic plague. A fortiori, the situation would be the same in the environment of patients with bubonic plague who are capable, in the septicemic phase, of causing the infection of the human ectoparasites responsible, when they proliferate, for the evolution of the endemia into an epidemic, according to the thesis of Blanc and Baltazard.

At the present time, vaccination is formally indicated only in the rural sectors where plague detection work reveals the existence of an enzootic relating to the domestic rodents, and where in the absence of adequate means of execution, systematic disinfestation is doomed to total or partial failure. In this respect, it is the epidemiological surveys that determine what steps must be carried out. The choice of vaccine is subject to a combination of local conditions, which does not exclude the psychological factor. For us, despite the progress made in increasing the protective value of killed vaccines, our preference is for live vaccines, and especially the EV vaccine which has stood the test of time and for which it would be desirable to undertake appropriate studies aiming at ensuring the stability of its characteristics.

While the physiognomy of human plague makes it possible to face the future with optimism, its total disappearance is a utopistic hope, and it is always necessary to maintain strict vigilance because new enzootic foci affecting many species of wild rodents have developed in the territories infected for the first time during the last pandemia, for example, the American continent, to mention only the most important of these areas.

Nobody can predict the duration of the dormant phase upon which we are now entering. The question has been extremely well treated by Baltazard [7] at the conclusion of 17 years of investigations which had yielded many data. These investigations were carried out in several Asian foci, notably in India. While our colleague is well founded in characterizing plague as "the disease of the future," because its virus is widely dispersed in nature, we are no less justified in assuming that the era of the hecatombs of past centuries and also of our century is over, because we now have at our disposal a means of etiological prophylaxis which can be readily applied and whose effectiveness has been demonstrated, with which we can prevent the infection of domestic rodents; and it is this infection which controls the endemia, the origin of all epidemic escalation.

However, the respite which nature is now granting us and which is powerfully supported by our prophylaxis, does not terminate the activities of research scientists. As a result of the immunization against plague, the efforts of researchers are directed at obtaining a preparation whose value will be at least equal to that of the best live vaccine. Will this objective ever be attained? We hope so, but we also hope that it will not be forgotten that the difficulties of protecting man by means of vaccination have been underestimated for a long time, and that failures are not exceptional even with the EV virus-vaccine, and finally that even the disease itself does not always impart a prolonged immunity because relapses of bubonic plague have been reported many times. Dieudonne and Otto, quoted by Pollitzer [215] have reported "cases of reinfection of persons who had previously contracted a plague which was bacteriologically confirmed, and maintained that, in general, the immunity acquired under natural conditions against this disease was evidently a relative matter, both as to its stability and its duration." These are precisely the two qualities for which we value the EV virus-vaccine so highly.

Despite a certain degree of reluctance which may be triggered by the principle of its use in man, nobody has so far denied the high experimental protective value of this vaccine. In the first part of this paper we have talked at length about the severity of the test to which our guinea pigs, vaccinated by a single inoculation, were subjected at varying intervals, and about their mode of reaction depending on the

degree of their immunity. On the basis of this behavior, the EV virus-vaccine could represent a reference test, a control which would permit making instructive comparisons in order to evaluate the qualities of a vaccine preparation under study. The initial attempt to achieve this was made by Keppie et al. [84] who obtained, by the repeated action of ultrasound on cultures of *P. pestis* -- which had been treated by an appropriate technique in order to eliminate the toxic fractions -- a residue free of all live elements, a residue which would seem to represent the antigenic complex most favorable to the protection of both the mouse and the guinea pig; these animals could apparently be immunized against 100-1000 lethal doses of *P. pestis*. Knowing from our papers that no guinea pig vaccinated by two injections of any killed vaccine has ever resisted our virulence test, regardless of the activity of this vaccine in mice, the authors asked us to test their preparation on the guinea pig according to our technique. To this end, H. Smith sent us the necessary equipment with a detailed experimental procedure comprising two subcutaneous injections at 14-day intervals, the animals having to be tested one week later. For comparison we prepared a series of guinea pigs treated with a single inoculation of EV vaccine. Furthermore, we agreed to set apart another group of vaccinated guinea pigs which were to be tested two months after the vaccination. Each lot contained five animals and an equal number of non-immunized controls.

The balance sheet of the operation may be drawn up as follows:

	"Ultra-sonated" vaccine	EV virus-vaccine	Controls
Test after 1 week....	3/5 (a)	5/5 (c)	0/5
Test after 2 months..	0/5 (b)	5/5 (d)	1/5 (e)

The numerator indicates the number of survivals:

(a) The three surviving guinea pigs showed an intense local reaction which regressed after 10 days. As for the two animals which did not resist, one died of acute plague, the other of subacute plague.
(b) The 5 animals died of acute plague as did the controls.
(c) There was no local reaction.
(d) Slight reaction for 3; more marked reaction for 2.
(e) The survival of a control which reacted with a voluminous local abscess is probably attributable to a latent or ancient infection by *Past. pseudotuberculosis* which entails a certain degree of resistance to the plague infection. This observation, which is not exceptional in Paris,

was never made on a control guinea pig in Madagascar, where pseudotuberculosis is unknown.

All guinea pigs which resisted the infection were sacrificed after two months; they were in a perfect state of health, and showed no residual lesion.

Despite the fact that in this comparison the virus-vaccine came out best, we informed our colleague that this was the first time that, under the conditions of our controls, we have seen any guinea pigs survive this test after immunization with a preparation devoid of live germs; hence, we added, the "ultrasonated" vaccine represents a notable advance over the other, totally or partially killed vaccines which we had had the opportunity to administer to the guinea pig. Subsequently, Cocking et al. [85] have elaborated on the considerations which had led them to the manufacture of this type of vaccine which they planned to use in humans. However, Chen and co-workers [23] have recently reported, on the basis of an argumentation which differs from that of the preceding authors, that a total formol-treated vaccine to which an oily adjuvant (mentioned above) had been added gives better results in the guinea pig than the "ultrasonated" vaccine. It would have been interesting to compare this vaccine with the EV virus-vaccine, using our experimental procedure whose results as we shall recall, permit three graduations in the interpretation of the animals' responses: total immunity (TI), partial immunity (PI) and absence of immunity (AI).

In an article written in 1936 for the general public [110], Prof. Pasteur Vallery-Radot referred to our work and to the prospects opened up by the use of a preventive vaccination against plague by means of a live vaccine, which at that time had aroused some interest in the medical world. After stressing that Yersin had been the originator of this idea some 40 years earlier but that this idea had not been followed up, the author concluded his article as follows: "The germ, whose virulence is spontaneously attenuated by nature or deliberately disciplined by man, no doubt creates a more stable immunity than the microbial body which has stopped living: life must be preferable to death."

These lines were written 25 years ago. For us, they are just as valid today as they were then, and we shall end our paper on this concluding note.

SUMMARY

1. In the first part of our paper we discussed the reasons which had led us, in 1926, to consider the substitution of a live vaccine (virus-vaccine in the Pasteurian sense) for the usual vaccines in the vaccinal prophylaxis of plague in Madagascar. These reasons were the manifest lack of success

with killed vaccines; the epidemiological circumstances specific to the Plateaux region of Madagascar with the constant threat of epidemic pneumonic plague, and especially the impossibility of conferring an appreciable immunity on the guinea pig by means of a killed vaccine, a fact which, moreover, had been admitted by all researchers. As it happened, we had many times noted in the laboratory that if a guinea pig resisted the inoculation of certain strains which had lost a part of their pathogenic potency, this animal subsequently enjoyed total immunity. One of these strains, called EV, appeared to us after five years of monthly subculturing at laboratory temperature (20-25°C) to possess the two properties required of a virus-vaccine: innocuousness and effectiveness, the latter reflected especially by the duration of the protection conferred by a single inoculation.

The application of this virus-vaccine to man, cautiously attempted by Robic in 1932, was gradually extended to the entire population exposed to the risk of infection, or about one million persons. As soon as the massive vaccinations had been carried out -- they were favorably accepted by both Europeans and Malagasy -- the plague mortality rate considerably decreased, which could be attributed solely to the vaccination, because the etiological prophylaxis was practically ineffective under the conditions in which it was carried out; some observations made under various circumstances are of an experimental value from this point of view. Vaccinations and revaccinations entailed only mild reactions. These vaccinations and revaccinations were continued until 1959, but the systematic disinfestation inaugurated about ten years previously and the general decline of the incidence of plague all over the world have considerably reduced the role of vaccinal prophylaxis in the fight against plague. We stressed how an analogous undertaking in Java by Otten, using another attenuated strain (Tjiwidej) had made it possible to strengthen our position adopted in Madagascar, due to the fact that the Dutch scientist has had the opportunity to prove, by a so-called alternating pilot experiment, the superiority of the live vaccine over the killed vaccine (Haffkine's lymph). More than one million inoculations were carried out in Java between 1934 and 1937 with results comparable to those recorded in Madagascar: in both places the number of cases of bubonic plague was reduced by 80 percent. Without claiming that the EV virus-vaccine gives a definite protection against primary pneumonic plague -- even though the experiments in guinea pigs did give an indication of such a protection -- the regression of pneumonic plague was parallel to that of the bubonic form, because the latter form is always at the root of the pulmonary complications which trigger the epidemic process by interpersonal contagion. In the meanwhile the laboratory studies led us to define the

characteristics of the EV strain and, as a result, define the characteristics which ought to be present in all other strains capable of being used in the form of a virus-vaccine; these characteristics are: the persistence of a residual virulence attested by splenic reactions in the guinea pig subjected to inoculation with large doses, and the persistence also of a toxic power linked with this low degree of virulence. However, of several cultures of this type, EV was the only one which gave the guinea pig an immunity (which was still appreciable after several months) against a very severe virulence test.

The antigenic value of the strain was made use of since 1933 for the preparation, by intravenous hyperimmunization of the horse, of a therapeutic serum whose quality was in no way inferior to that which would have been obtained with virulent germs; the latter, however, could not be handled without risks.

The interest aroused by the new vaccine stimulated new studies in foreign government laboratories to which the strain had been sent; these studies confirmed the data acquired in Madagascar both on the theoretical level and in the practical field of application. Stress was placed particularly on the studies of the Russian authors who, for almost 30 years, have been making a major contribution to the study of antiplague vaccination using live microbes, and notably the EV virus-vaccine whose high protective power is emphasized in their publications.

II. In the second part, the relationships between the immunological processes and the EV virus-vaccine are analyzed and discussed as a function of the modern researches on the antigenic structure of *P. pestis* which had been unknown prior to 1945. The circumstances arising from the Second World War have been the starting point of a large number of investigations aiming at the isolation of the protective antigens for the preparation of vaccines destined for troups which were called upon to stay in or travel through the African or Asian areas known to be foci of endemic plague. While recognizing the value of the live vaccine whose application in Madagascar became necessary on account of an exceptional situation, the officers of the Anglo-American medical corps never considered the use of the live vaccine.

In San Francisco, a team of American workers under the direction of K. F. Meyer isolated a water-soluble antigenic fraction -- F 1 -- in the crystalline state; this substance is devoid of toxicity, has a high protective effect in the mouse, but not in the guinea pig. Subsequently the authors re-examined this assertion, noting that the addition of an oily adjuvant to the F 1 solution elaborated in the cultures incubated at 37°C -- the temperature most favorable for the production of the capsular substance of which F 1 is one of the constituents -- made it possible to protect the guinea pig as well. Since then

the immunizing power of a vaccine, whether killed or live, was considered by them to be a function of the F 1 content of the strain. This simplification of the immunogenic process, combining into a single mechanism the action of a killed vaccine and that of a live vaccine, did not agree with what we had learned from our experiments with the EV virus-vaccine. In this connection, we have to recall a few definitions relating to the terms virulence, toxic power, and pathogenic power, whose interpretation -- different from that adopted in France after Maurice Nicolle -- was a source of confusion. The notion of attenuated or weakened virulence was absent from the American studies which knew only of virulent or avirulent strains. Now, strain EV is not avirulent, and our colleagues from San Francisco took this into account, because they confirmed and went beyond our old observations by reporting that the components of our vaccine showed a transitory proliferation in the animal organism while we had only demonstrated their existence in the organs of the guinea pig several days after a subcutaneous inoculation, without having had any grounds for speaking of proliferation; the latter, although temporary -- showing specific reactions which were defined after histological examination by Bablet and Levaditi -- cannot be interpreted in any other way but as a manifestation of a certain degree of virulence. It is this persistence of live microorganisms within the lymphatic tissues for which *P. pestis* has an elective affinity that is, for us, the essential factor which determines the rapidity and duration of the protection conferred on the animal by the virus-vaccine, while the preparations devoid of live cells require at least two injections at 8-15-day intervals in order to confer an immunity whose duration has never been specified because the tests generally took place during the two-week period immediately following the vaccination. As for the presence of seral antibodies whose measurement may perhaps permit an estimation of the antigenic potency of the various killed vaccines, it has no relationship with the quality of the immunity which may be attributed to the live vaccine, an immunity which, in plague infection, is primarily of a cellular nature. It is the increased phagocytic capacity of the macrophages and microphages that conditions the degree of protection following the vaccination, and there is no doubt that this process develops to the greatest extent in the lymphatic organs in contact with the live germs, just as it takes place in the defense of the organism against natural plague infection.

It was the studies of Burrows and Bacon at Porton Laboratory that convinced us of the originality of the EV strain, and permitted us to find an explanation for its powerful immunizing power which had been noted 25 years previously by our experiments in the guinea pig. By proceeding to the study of the factors responsible for the virulence of *P. pestis*, of

which precipitation-diffusion in an agar medium brought to light two special lines attributable to an antigenic couple ? - VW which to a large extent determined the resistance of the coccus bacillus to phagocytosis, the authors were surprised to note the existence of the complex VW + in a single one of the so-called protective avirulent strains (actually, attenuated strains), namely strain EV 76. Other characteristics inherent in the virulent strains are also detectable in the EV strain, but we tend to think that the strain, which exhibits so many similarities with the virulent strains without having the latter's pathogenic power, owes the permanence of its characteristics which were unchanged for 30 years to the conditions at which we had preserved it in the laboratory. We have dwelt at length on the importance of factor VW, despite the fact that the English-speaking authors refuse to admit that it plays a protective antigenic role. Comparison of the Tjiwidej strain and the EV strain revealed the existence of profound differences between the two when mice are subjected to the action of cortisone or to iron salts prior to immunization, substances which have an inhibiting effect on the operation of the reticulo-histiocytic system: under these conditions EV apparently becomes pathogenic for mice, while Tjiwidej does not. Certain considerations relating to two other attenuated strains whose behavior in the guinea pig was found, in recent experiments, to be similar to that of the EV strain, justify our point of view.

A phenomenon of homologous interference between EV and a virulent strain was discussed from the point of view of the various related hypotheses, notably that of specific and non-specific immunity.

Because of the heterogeneity of the *P. pestis* cultures and of the cellular dissociation which may produce the more or less rapid degradation of the antigenic power of strain EV, its method of preservation is not a matter of indifference, and all possible precautions should be taken to prevent this dissociation. However, animal passage would make it possible to recover a regenerated culture without the latter having become pathogenic by this process.

While today antiplague vaccination is only of secondary interest, and we have stated why this is so, the mechanism of the immunity conferred by the various types of vaccines continues to be a matter of current interest on the level of general immunology. The nature of the protective antigens has again been called in question by the recent studies of the Porton team.

Without ignoring the importance of the data obtained from the antigenic analysis which, prior to the employment of the techniques of precipitation-diffusion, has revealed the existence of an unsuspected complexity of the antigenic system

of *P. pestis*, in the final analysis it is the animal experiments which retain primacy in this area; it is these experiments which, apart from any theoretical considerations, have formed the basis of our studies and of the applications which these tests have sanctioned. The vaccination by a live vaccine and especially by the EV virus-vaccine will have marked a stage and signified an advance in the protection of man against plague.

Acknowledgements

We wish to express our deep gratitude to Dr. K. F. Meyer for the interest which he has shown in our studies and for his kindness in placing at our disposal a supply of fraction 1 as well as the plague strains A.1122 and F.7793-10; and to Dr. T. W. Burrows who has rendered us the most valuable assistance in the antigenic analysis of the strains which we have sent to him.

APPENDIX

Production of Crystalline Concretions of Proteinic Nature by the EV Strain of *P. pestis*

During 1933, the attention of our collaborator J. Robic was captured by an anomaly which occurred in the cultures of strain EV which had been subcultured in the laboratory of E. Dujardin-Beaumetz at the Pasteur Institute of Paris. He noted the appearance of whitish concretions which dotted the microbial layer on the surface of the sloping agar, and which at first sight could have been taken for contaminating molds. However, this was not the case, and microscopic examination showed that these new formations were composed of crystalline needles and of plague bacilli which could be readily subcultured in the pure state, after which the phenomenon tended to recur when the cultures were 2-3 weeks old. Nothing of the sort occurred at Tananarive where strain EV had been maintained by regular subculturing for seven years on sloping agar. Under these conditions it had to be assumed that 1) for the time being the medium used at Paris was the only one favoring the appearance of this phenomenon; 2) that this phenomenon was specific to the EV strain, because it had never been observed by Dujardin-Beaumetz who had cultivated and preserved strains of plague bacilli on this medium for a long time.

Robic had noted that the appearance of the crystals was partly linked with a certain degree of desiccation of the medium, because if the tubes were sealed after a sufficient development of the culture, the latter retained its normal appearance. He collected tubes which showed a more or less abundant formation of these concretions, according to whether they had been sealed for a longer or shorter period of time. However, this phenomenon did not take place consistently, and it could not be reproduced in series; under apparently identical conditions the subcultures originating from "crystal-containing" cultures did not always produce it. The harvesting of these crystals was a laborious task because of their deep adherence to the medium: samples taken by means of a spatula contained both plague bacilli and agar particles. Nevertheless, we were able to collect a sample of this material in 1936 and this sample was sent by E. Dujardin-Beaumetz to Professor G. Bertrand who detected in it the presence of phosphorus, magnesium and a nitrogen-containing compound which showed the characteristics of trimethylamine; however, the operation was carried out on a much too limited amount of sample to enable the eminent chemist to carry out a more detailed analysis.

Here the matter rested for a while; furthermore, we did not consider it opportune to draw attention to a transitory anomaly observed on our virus-vaccine strain at a time when human vaccination was our primary concern in Madagascar. Let us say that the phenomenon was no more than a bacteriological curiosity which did not in any way affect the properties of the EV strain.

However, we occasionally noted, in Paris, the appearance of crystals on old cultures grown on ordinary nutritive agar, preserved in the refrigerator, and Robic had made the same observation in Tananarive; from our collaborator we received a tube of culture in deep agar whose surface was entirely covered with crystals. Circumstances had led us to study the value of various media for the cultivation and preservation of our plague-bacillus strains and, in particular, of the EV strain; and it was by accident that we noted, in 1947, that all colonies of this strain, isolated on a certain medium, were covered with a sort of rime which appeared within 20 days; in this rime we found the crystalline needles inside a mass of normal plague bacilli. By their appearance, these colonies offered a striking contrast to those which had grown on other media simultaneously seeded with the same sample of EV. This medium was a cold macerated homogenate of fresh beef (the heart was not suitable) treated with 20 g of commercial peptone (type "Uclaf") and with 5 g of NaCl per liter, with agar being added to 20 parts per thousand, and the final pH adjusted to 7. The crystals formed equally well on the agar surface in the tube and in Roux's glass box. From that time on we were able to reproduce this phenomenon and carry out its study.

We then recalled that the medium used in the laboratory of Dujardin-Beaumetz consisted of a cold macerated homogenate of beef followed by peptonization of this same meat by the gastric mucosa of the pig. Thus, the characteristic common to these two media -- his and ours -- was the maceration and not the peptone, because the "Uclaf" peptone could be replaced by a casein peptone without interfering with the formation of the crystals in some experiments; however, afterwards we used solely the first medium of which we had an ample supply reserved for this use. For four years, despite several failures which are still unexplained at this time, we were able to maintain a collection of "crystalline" cultures by multiplying our subcultures on this medium, and to report, in 1952 [71], our first findings on this curious phenomenon:

"1) The new formations are composed of true birefringent crystals and of plague bacilli. Their color is mostly chalk-white, sometimes yellowish; they are never observed on non-seeded control tubes, regardless of their degree of desiccation.

2) Their transfer to a new medium always gives a normal *P. pestis* culture, with all the properties exhibited by the EV strain.

3) The vitality of the cultures associated with these crystals is at least equal to and often greater than that of cultures which do not contain crystals, as is proved by the subcultures carried out with both types after several years of preservation in the refrigerator.

4) Three clones derived from cells A, B, C, isolated by Tchan in 1949 [144] by means of the micromanipulator give rise to crystals in the same way as does the sample of EV strain from which they originate.

5) Two avirulent and one virulent strain of *P. pestis* and a strain of *P. pseudotuberculosis*, cultivated on the medium defined above, show no crystallization after several weeks, while the EV strain exhibits crystallization after the 15th day."

At that time, the nature of these concretions was unknown to us, but, let us add, the numerous French and foreign microbiologists who had the opportunity of visiting our laboratory concurred in stating that no new formation of this macroscopic appearance had ever been observed on any microbial culture. As far as we know this is still the case today.

Our first objective was to collect a sufficiently large amount of material so that we could proceed to its investigation. In reality, although for several months we were in a favorable position because we had an almost continuous production of crystals on the above-mentioned medium, we noted that this production was subject to capricious variations, and it was only after a long time that we were able to determine the conditions most favorable for their production. Thus, the seedings must be carried out on dry media, without water of condensation; then it is necessary to start from cultures already containing crystals, in which the microorganism remains viable despite the fact that the cultures had been preserved for several months at room temperature; a concretion is transplanted into peptone-wafer where it crumbles when scratched with a spatula; the plague bacillus is grown in this medium in 2-3 days in the oven at 25-28°, and it is from this culture that a loop carefully spread on the surface of the agar is removed. If we use Roux's glass box, the seeding is carried out with 4-5 ml of the culture in peptone water which will be quickly absorbed by the dry surface of the agar after a few to and fro movements executed in order to cover this surface uniformly. The tubes and boxes are incubated at 25-28° for 4-5 days; the culture is already quite visible; they are then placed, uncapped, in a chest maintained at the temperature of the laboratory. Fifteen-twenty-five days later in the case of the tubes, and 3-4 weeks later in the case of the boxes the

concretions appear on the upper part of the medium where the agar is thinnest and driest, and then gradually spread over the whole surface, or remain limited to the upper quarter of the medium, without our being able to predict which of these two modalities will be adopted by the process. It is a curious and consistent fact that a series of tubes or boxes will always have the identical appearance after a seeding carried out simultaneously with a given suspension, which in no way prejudges the events which are observed in another series with cultures originating from these tubes (Figs. 5, 6)*.

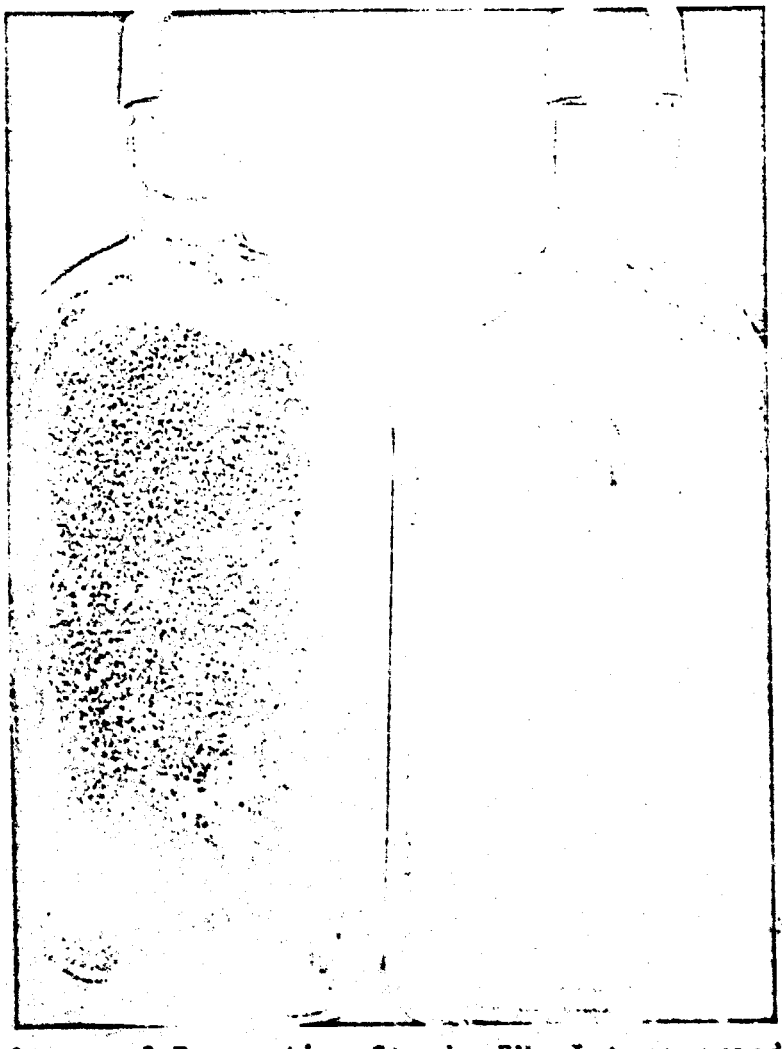

Fig. 5. Culture of *P. pestis*, Strain EV, Interspersed with Crystals. On the right, control culture of the same strain on peptone agar.

*The photographs illustrating this chapter were made by the Photomicrography Service of the Pasteur Institute, under the direction of P. Manigault, to whom we want to express our thanks for his valuable assistance.

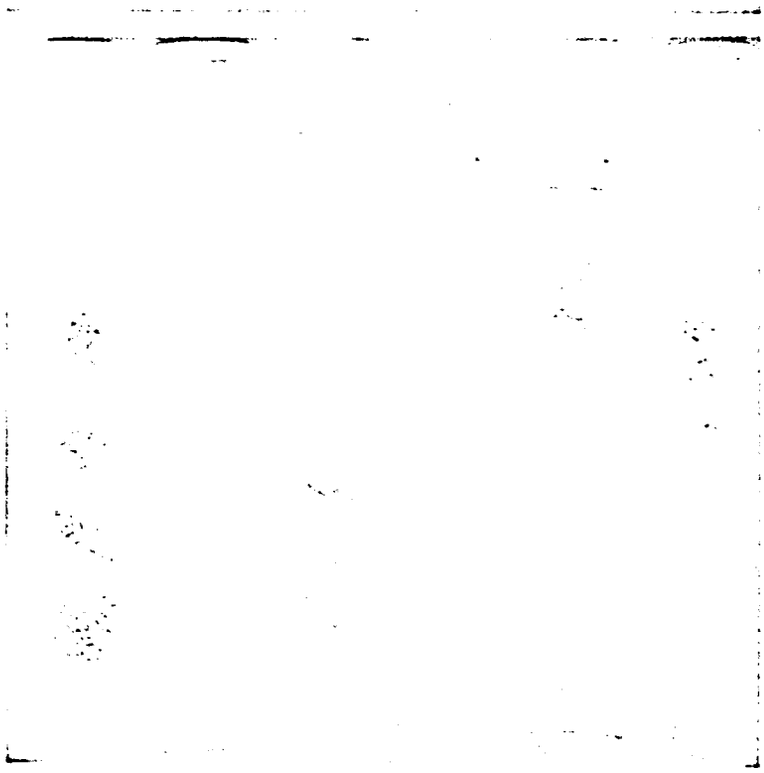

Fig. 6. Crystallizations in Culture Tubes of *P. pestis* EV, with Two Tubes of Control Tubes of Normal Culture. On the right, two tubes seeded over the entire surface, in which the crystallizations were limited to the upper part of the culture.

These formations are linked mainly to the development of the culture, but become visible only at the level of the colonies themselves; this is manifested on tubes comprising well-isolated colonies, even a single one (Fig. 7). This can be proved in a peremptory fashion as follows: Let us touch the surface of a tube of the broth-peptone (Uclaf) agar-containing medium with a trace of *P. pestis* agar culture which has been rendered lysogenic under the effect of a phage, after spreading on this surface a drop of a suspension of crystals and EV microbes; the lysogenic strain will develop discretely at the

point touched and will create within a radius of 3 to 6 mm around this point a zone which will be devoid of any culture; beyond this, both above and below this zone, the EV strain will multiply normally and the crystallizations, absent both on the lysogenic culture and around it, will appear where the growth of EV begins (Fig. 8). Although, as has been indicated by Robic, the desiccation is a determining factor of this phenomenon, since it is never seen in sealed tubes after a few days of cultivation, the substance which makes up these concretions forms also in liquid medium; hence, the agar plays no role here. Thus, a culture, which had developed abundantly under a thin medium in an Erlenmeyer flask for six weeks at 20-25°, was left in the oven at 37°C for several months. After evaporation, the sediment contained a large amount of crystals having the same appearance as that of the agar cultures. We have recently made this observation on tubes left uncapped in a chest for a very long time, although there is still a little broth left above the microbial deposit. Nothing of this sort has ever been observed in cultures of another *P. pestis* strain subjected to identical conditions for control purposes.

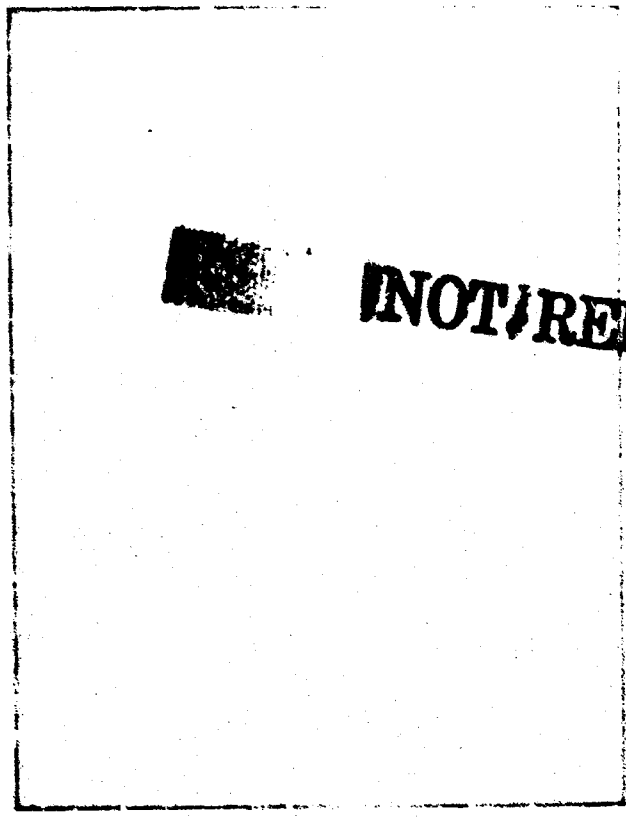

Fig. 7. Isolated Colonies of *P. pestis* EV, All Covered with Crystals. Note the absence of concretions outside the single wide colony in the left tube.

These new formations are of a variable consistency which depend on their age, because we have noted that they crumble more easily in the water of samples taken from older cultures than in that of younger cultures; however, this is not necessarily the case.

If, after grinding, one of these samples is suspended in physiological saline solution, the latter assumes the appearance of lime water; by contrast, in distilled water, the turbidity is much less pronounced, because the crystalline needles are soluble in it and only the plague bacilli incorporated in their mass remain suspended in this material whose crystals represent the dominant element. Hence, we were able to obtain, by filtration on a filter candle, a solution of these crystals devoid of microbial cells but nevertheless containing a part of their water-soluble substance as well as traces of agar. The addition of a saturated solution of NaCl to the filtrate re-precipitates them in a form that is agglomerated mostly into more or less voluminous spiny clusters (Fig. 9).

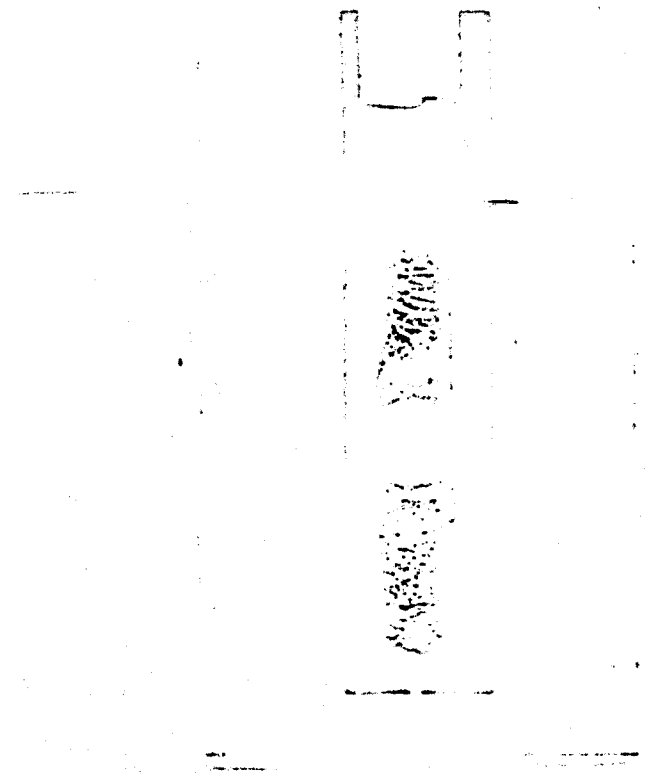

Fig. 8. Tube Seeded with *P. pestis* EV Over Its Entire Surface. A point in the center was touched with a trace of lysogenic strain. The latter developed by creating a lytic zone around itself. The crystals appeared only outside this zone where the EV strain was able to develop.

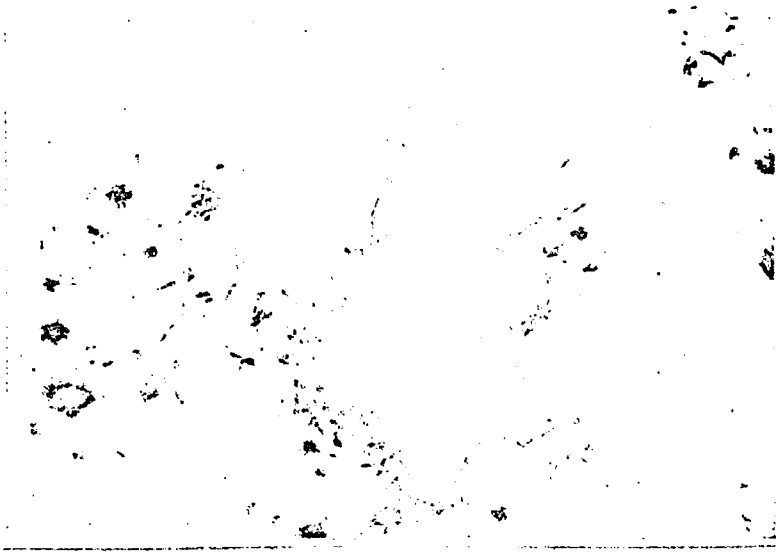

Fig. 9. Appearance of the EV Crystals (*P. pestis*) After Precipitation with NaCl from Their Solution in Distilled Water, Filtered Over a Filter Candle (magnification 500 x).

It is on this material that our colleages G. Milhaud and J. P. Aubert have carried out their first analyses whose results, which they were kind enough to communicate to us [72] we have reported elsewhere, and which we will reproduce here:

"The UV spectrum gives a 280 mμ/260 mμ absorption ratio of 1.8. On the other hand, after hydrolysis in HCl for 14 hours and subsequent paper chromatography, we have identified the following amino acids so far: aspartic and glutamic acid, alanine, leucine, isoleucine, valine and lysine. These two analyses permit us to conclude that the crystalline product is of proteinic nature." On his part, P. Manigault has examined the "crude" concretions with the aid of a microscope in polarized light and fluorescence and published his findings [97]. He noted that "the densest concretions may be resolved into homogeneous crystalline fragments the smallest of which always preserve the appearance of fine needles executing Brownian movements." His communication also reports a few physical constants of these elements which are all anisotropic and birefringent, and of which he has obtained nice photomicrographs (Fig. 10).

Fig. 10. Microphotograph of EV Crystals (*P. pestis*): objective 11; eyepiece 6 x; print reduced 50%. Magnification during photography: 30 x. Enlargement on paper: 250 x. Photographed in polarized light between crossed analyzer and polarizer, without perceptible gradation. (P. Manigault.)

The analyses were not carried out in greater detail, but they have nevertheless permitted to conclude that these concretions, which are constituted of real crystals, are proteinic in nature.

What is the process which causes this phenomenon and what is the meaning of this occurrence? Even its reproduction is shrouded in darkness because, as we have seen above, it upsets the predictions and only by carrying out many operations were we able to collect a sufficiently large amount of material

with which to carry out our studies*. There are notable differences in the capacity of the different subcultures to form crystals; this is particularly the case with EV samples received from other laboratories. On the other hand, in certain tubes covered with concretions we have repeatedly observed the development of voluminous thick colonies which are not infiltrated by crystals; these colonies subcultured by spreading them on a favorable medium never produced these crystals. They may be classified with Wake's "third colonial form" discussed above; they are usually observed in the case of *P. pestis* and indicate, by the loss of their antigenic power, the degradation of the original strain. Hence, it would seem that the maintenance of the integrity of the characteristics of strain EV is an indispensable condition for the appearance of the phenomenon. Let us recall that when this vaccinal strain was observed by Robic 28 years ago, it was at the peak of its immunizing power. Hence, the production of crystals ought not to be interpreted as a sign of degradation.

What is the extent of the participation of the very substance of the coccus bacillus in the elaboration of this protein which, because of its rapid precipitation by NaCl, must have a low molecular weight? Does the action of the microbe consist solely in exteriorizing certain polypeptides of the culture medium by a special process? For the moment these questions cannot be answered. The few tests which we have carried out in an attempt to demonstrate the existence of an antigenic relationship between these crystals and the EV strain resulted in failure, as evidenced by the absence of specific agglutinins and precipitins in a rabbit antiserum prepared by intravenous injections of their solution, and the absence of immunity in the mouse to which the solution was twice administered subcutaneously (to be sure, without oily adjuvant). Perhaps our colleage J. Fournier who succeeded us in the laboratory of plague research will derive some benefit from the material which we have left him, in order to continue this study which has only been outlined so far, and in which we know that he is interested.

When we state that a similar phenomenon has never been noted in microbiology in the form in which it presents itself and which we have described, we do not mean to say that this is the first time that crystalline needles were produced on nutritive agar by pathogenic microorganisms. Pfeiffer (1889), Parietti (1890), Preiz (1894), Delbanco (1896), quoted by H. Mollaret [102a] have noted the presence of small crystals around colonies on gelatin or in deep agar prepared by B. de

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*With the technical collaboration of Andre Chevallier and Madeleine Boutin, to whom we wish to express our affectionate gratitude.

Malassez and Vignal. There are no hints as to the nature of these formations which, at the time the observations were made, were interpreted in a questionable manner. Moreover, Dujardin-Beaumetz has never made such observations on the cultures of this microorganism which he maintained on the same medium as the one used for *P. pestis*. Nor has Mollaret observed the appearance of crystals on the 600 strains of B. de Malassez and Vignal investigated in his thesis.

In 1916 Papin et al. [109] reported the presence of small black traces in the cultures of meningococci, which they identified as being composed of crystals. They recalled that even Bettencourt and Franca (quoted by Mace in the sixth edition of his *Traite de Bacteriologie* (Textbook of Bacteriology), Vol. 1, p. 498 (1912) had made a similar observation. Cultures of typhus- and diphtheria bacteria were likewise said to have exhibited this characteristic. However, these "small black traces" were visible only under the magnifying glass, and appeared after 48 hours. For Papin et al., these crystals, which infiltrated the colonies by forming a series of dots readily visible when examining the colonies under the microscope at a low magnification, constituted a valuable element for the diagnosis of meningococci.

What comparisons can we make between these crystals and ours? First of all, there is an incontestable analogy between their respective mode of production, which takes place within isolated colonies; moreover, both are real crystals, because they exhibit the characteristics of crystals when examined under the polarizing microscope between two crossed Nicol prisms. However, they also exhibit a basic difference: the crystals which are isolated after the suspension of meningococcus colonies in water are insoluble in the usual solvents and notably in water; the action of sodium hydroxide made these crystals more apparent, and they seemed (sic) to resist the action of HCl. Moreover, their rapid appearance in the cultures contrasts with the delay of at least 15 days required in the case of the EV strain. Despite the intention expressed by the authors of undertaking the chemical study of these new formations which appear under the microscope as fine crystalline needles, we have found no indication which would shed any light on their composition. Apparently this property associated with the meningococcus, with the practical significance assigned to it by Papin et al., did not retain the attention of the bacteriologists, because it is passed over in silence in the curriculum of the Pasteur Institute. We could not, in the framework of this paper, fail to rescue this fact from oblivion.

However, the phenomenon inherent in strain EV has permitted us to throw some light on the nature of a strain of

P. pestis isolated in 1950 in the former Belgian Congo under a priori strange circumstances. It was only four years later, after much hesitation for which we were partly responsible, that Jesierski, Fain and Devignat [82] issued a communication which contained the following summary: "The authors have isolated in Elisabethville, from a sick horse, a strain of *Past. pestis*, var. *orientalis*, which exhibited an attenuated virulence and possessed vaccinal properties. This strain differs from all other strains isolated until now in the Belgian Congo in the rural foci of the Ituri and Kivu, which are of the *antiqua* variety. Moreover, the distance between these two foci is more than 1500 km, as the crow flies." The above-mentioned strain had been obtained by Jesierski from the nasal mucus of a saddle horse born in Elisabethville, a city which this horse had never left, and which was slaughtered for subsequent delivery to the butcher trade, because it had exhibited a "tumefaction of the cranial framework which, it was believed, could be attributed to osteoporosis."

The germ was identified only after Jesierski had sent the culture to the Blukwa plague laboratory. No doubt the strain, a subculture of which had been sent to us by Devignat, had indeed possessed all the characteristics noted at Blukwa.

This discovery of a plague bacillus in a region where plague had been unknown at all times (this is still the case today) in a horse, a species which is refractory to both the natural and experimental infection, and the fact that the strain belonged to the oceanic variety (Devignat's *orientalis* variety) when all the strains of *P. pestis* in the Congo had been found to belong to the continental variety (Devignat's *antiqua* variety) represented an enigma for us. From the very beginning we found so many analogies with the EV strain that we made a comparison and soon concluded that the two cultures were identical [73]. In effect, Burrows, whose competence we relied on in the antigenic study of the Elisabethville strain, identified, in the latter, the complex VW which of all attenuated strains is present only in the EV strain as we have stressed above; moreover, taking into account the other shared characteristics, Burrows informed us that he had reason to believe that "Elis" (abbreviation of Elisabethville) and EV were strains that were intimately related to one another, one being a derivative of the other. Now, by a lucky accident -- because we know the capriciousness of the production of crystals by the EV strain -- these concretions appeared in their usual form in two tubes of "Elis" culture. Thanks to this decisive criterion, there could be no more doubt about the identity of the two strains. Since the EV strain, the only one belonging to the *orientalis* variety, had been maintained at the Blukwa laboratory for the preparation of virus-vaccine and, on the other hand, since it was confirmed that no experiment had been carried out with it on the horse in question, it seems logical to

conclude from this curious story that the Elis -- alias EV -- strain could only have resulted from an error, and it was not our concern to look for the origin of this error.

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